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XXIV. STUDIES ON COAGULATION. I. ON THE VELOCITY OF GELATION AND HYDROLYSIS OF GELATIN SOL.

By RINNOSUKE SHŌJI.

(*From the Physiological Institute, Imperial University of Kyoto, Japan.*)

(*Received June 4th, 1919.*)

I. The Gelation Velocity of Gelatin Sol.

Gelation of hydrophilic colloids has frequently been studied in regard to its 'gelation time' (usually called the coagulation time) *i.e.* the time necessary for the gelatinising of a sol. The definition of 'gelation time,' however, is somewhat arbitrary and differs with the observer. Some observers consider a sol is gelatinised, when the vessel containing it can be inverted without spilling. Others consider it gelatinised, when it adheres as an amorphous mass to a rod inserted in it. Still others consider it gelatinised, when a small glass bead thrown into it stops half-way down. Gelation, however, is not a sudden change of state, but a kinetic process with respect to time. Therefore it is more important and fundamental to study the course of a whole process of gelation than to study simply the gelation time.

It is not yet known exactly, what change of state the colloidal constituents undergo in the process of gelation. The process is called gelation only when a hydrophilic sol, separating into two phases, gradually increases its viscosity until it becomes an amorphous solid mass throughout. Thus the degree of gelation can be estimated by measuring the increment of viscosity, and the rate at which the viscosity increases is called the 'gelation velocity.'

Von Schröder [1903] studied this problem as follows. He heated a gelatin sol at 100° for a certain time, and then cooled it suddenly to 25°. He now kept the sol at this latter temperature, and measured its viscosity twice, first, after a lapse of five minutes, and again, after sixty minutes. The second value was of course larger than the first one. He called the difference of these two viscosity values $\Delta\eta$, and the time interval between the measurements (55 minutes) Δt, and assumed that $\frac{\Delta\eta}{\Delta t}$ represented the coagulability of the sol. He found that the longer the sol was kept at 100°, the smaller were both the viscosity at the first measurement and $\frac{\Delta\eta}{\Delta t}$.

Levites [1908] later studied this problem in the same way. He measured the viscosity of a sol several times in the course of its gelation, and found that the gelation velocity, $\frac{d\eta}{dt}$, was a constant independent of the time, so that the viscosity is a linear function of the time. He studied the process, however, only during the first 1·5 hours of a gelation, which is a relatively brief period when it is remembered that the whole process, according to his own statement, occupied from 11 to 24 hours. His conclusion, therefore, is necessarily somewhat insufficiently supported by experiment.

The present writer took the same problem. The method and procedure followed by him were as follows. A weighed amount of dried commercial gelatin is put into water in such proportions as to have a desired percentage solution when dissolved. This mixture is kept in a cool place in a flask for a certain time in order to allow the solid gelatin to swell sufficiently. It is then placed in a thermostat at 70° or 80°, and by thorough shaking is brought to a homogeneous sol in a few minutes. The flask is fitted with a condenser to prevent the sol from evaporating. At successive intervals after the beginning of heating 5 cc. of the sol are taken out of the flask, and put into an Ostwald viscosimeter kept in another thermostat at 18° and allowed to gelatinise at this temperature. Its viscosity coefficient is then determined frequently over a considerable period of time. The first determination is made at the end of the first six minutes, preliminary experiments showing that the temperature of the sol brought into the 18° thermostat falls to this temperature in five minutes. The initial viscosity coefficient, *i.e.* the viscosity coefficient at the initial time of gelation is found by extrapolation, assuming that the process of gelation begins regularly as soon as the sol is brought into the 18° thermostat.

Since the successive portions are taken out of one and the same sol, they differ only in the length of time they have been previously heated, and the whole course of gelation of each of them is studied in detail, since it is desired to study the influence of previous heating on the process of gelation, as well as the process of gelation itself. The observations give the relative viscosity coefficient, that of water at 18° being taken as unity. The absolute viscosity coefficient can then be easily calculated if desired.

The results of experiments with a 3 per cent. gelatin sol are shown in Fig. 1. The quantities $\eta - \eta_i$ are taken as ordinates and the time t as abscissa, η and η_i being the viscosity coefficients at the time t and at the initial time of gelation respectively. Curves 1, 2, 3, 4, 5 and 6 are viscosity-time curves for portions previously heated for six minutes, 1, 3, 6, 11 and 20 hours respectively at 80°. Fig. 2 is another example of a 3 per cent. sol which was heated at 70° instead of 80°, curves 1, 2, 3, 4, 5, 6 and 7 corresponding to portions heated for 6 minutes, 2, 6, 16, 20, 30 and 39 hours respectively.

It is seen clearly from these figures that the longer the sol is previously heated, the smaller is the initial gelation velocity, a result in agreement with

that found by von Schröder. This disagrees, however, with Levites since the viscosity fails to be a linear function of the time in all cases. When the sol is heated for a relatively short time, the gelation velocity, $\frac{d\eta}{dt}$, increases with the time, so that the curve is convex towards the t-axis (Fig. 1, Nos. 1 and 2). For the sake of convenience let us call this the first case. On the contrary,

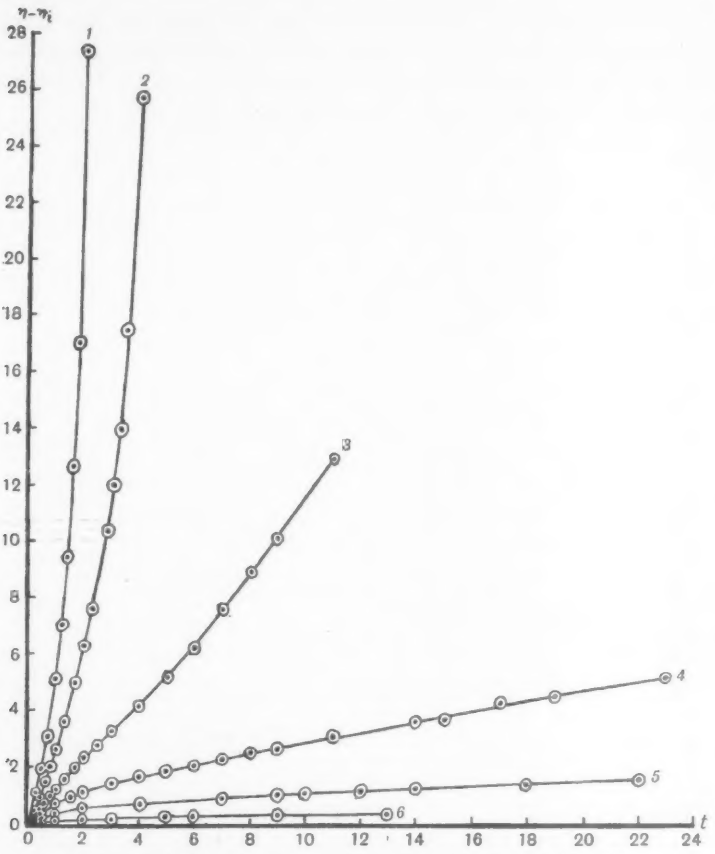

Fig. 1. Gelation of 3 per cent. gelatin sol. (previously heated at 80°).

when the sol is heated relatively longer, the gelation velocity decreases with the time, so that the curve is concave towards the t-axis (Fig. 1, Nos. 4, 5 and 6), which we may call the second case. At some intermediate 'critical' stage the gelation velocity is constant, so that the curve is a straight line. Fig. 1, No. 3 approximates to this case.

It is generally believed that α-glutin, the actual gelatinising constituent of gelatin, is gradually hydrolysed, by heating, to β-glutin which has no tendency to gelatinise even at low temperatures, and as a matter of fact, the longer the sol is heated, the further the hydrolysis goes. Adopting this point of view, therefore, we may say that the higher the concentration of α-glutin, the larger the initial gelation velocity; that when its concentration is higher than a certain critical value, the gelation velocity increases with the time until the viscosity grows beyond bounds; and that when the concentration is lower than this critical value, the gelation velocity decreases with the time, until the viscosity ultimately reaches a finite, limiting value. It is not clear whether the concentration of β-glutin affects these facts or not.

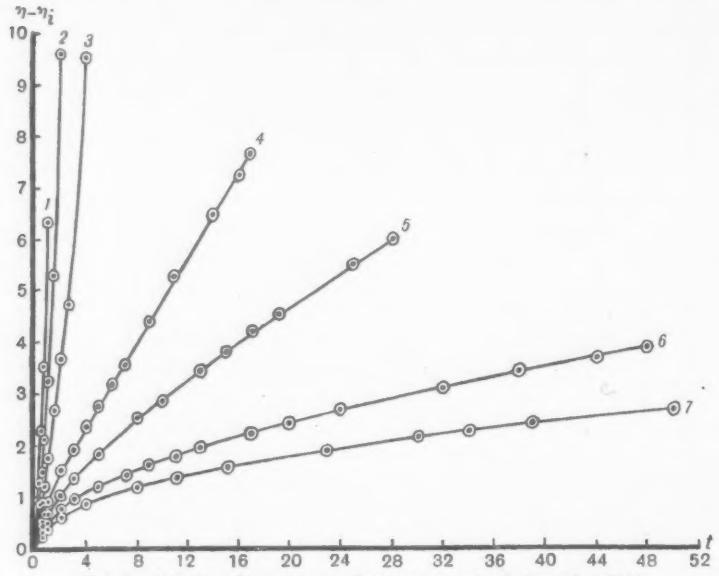

Fig. 2. Gelation of 3 per cent. gelatin sol. (previously heated at 70°).

These conclusions are supported by similar experiments with a 1 per cent. gelatin sol represented in Fig. 3. Curves 1, 2, 3 and 4 are viscosity-time curves for portions previously heated at 80° for 0·1, 1·2, 4 and 11 hours respectively. We see that the gelation velocity decreases with the time even when the sol has been heated for only a short period. That is to say, the gelation belongs to the second, and never to the first case. Obviously the concentration of α-glutin in this case has been lower than the critical concentration from the beginning.

The numerical value of the critical concentration of α-glutin cannot be found, since we do not know the concentration of α-glutin in solid gelatin.

The curves of Figs. 1, 2 and 3 suggested the empirical equation

$$\eta - \eta_i = \frac{\mu t}{1 + \frac{\mu}{\lambda} t} \quad \text{.....(1)}$$

both μ and λ being constants. To test this equation we may write it as

$$\frac{1}{\eta - \eta_i} = \frac{1}{\mu}\frac{1}{t} + \frac{1}{\lambda} \quad \text{.....(2)}$$

a linear equation in $\frac{1}{\eta - \eta_i}$ and $\frac{1}{t}$. It is then found that equation (2) is a good fit. The straight line (the figure is not given here) for the critical case $\lambda = \infty$ passes through the origin in each case, the straight lines below it ($\lambda < 0$) corresponding to samples heated for a shorter period, and the straight lines above it ($\lambda > 0$) corresponding to samples heated for a longer period.

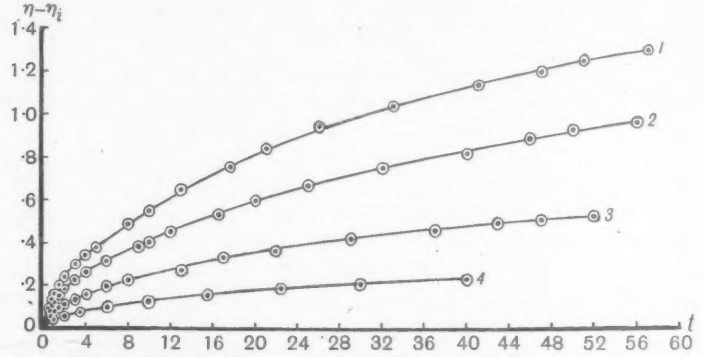

Fig. 3. Gelation of 1 per cent. gelatin sol. (previously heated at 80°).

The constant μ is plainly the value of $\frac{d\eta}{dt}$ at $t = 0$, *i.e.* the initial gelation velocity; we may regard it as increasing with the concentration of α-glutin. It ranges from 0 to ∞. As to the constant λ we may regard it as increasing with the concentration of α-glutin up to the critical concentration where it becomes infinite. For slightly higher concentrations λ is large and negative and decreases through negative values to a definite limit. This is more or less equivalent to regarding $\frac{1}{\lambda}$ as given by $\frac{1}{c} - \frac{1}{C}$, where c and C are respectively the concentration of α-glutin and the critical concentration. In terms of the curves we may say that λ, when positive, is the value of $\eta - \eta_i$ attained after a long interval of time, while, when λ is negative, the $\eta - \eta_i$ increases beyond bounds after a time given by $t = -\frac{\lambda}{\mu}$. In this latter case it is reasonable to speak of a 'gelation time' but not in the former case. As we see from equation (2) the constants $\frac{1}{\mu}$ and $\frac{1}{\lambda}$ may be taken directly from the straight lines of its graph; $\frac{1}{\mu}$ represents the tangent slope and $\frac{1}{\lambda}$ the intercept on the vertical axis.

Equation (1) may be looked upon as the solution of the differential equation

$$-\frac{d}{dt}[\lambda-(\eta-\eta_i)]=\frac{\mu}{\lambda^2}[\lambda-(\eta-\eta_i)]^2 \quad \text{.....(3).}$$

Now it may be shown that not only the equation (2), but also the solutions of the equations of more general type

$$-\frac{d}{dt}[\lambda-(\eta-\eta_i)]=\frac{\mu}{\lambda^n}[\lambda-(\eta-\eta_i)]^n,$$

where n is an integer, represent curves of the same general character as those given by the above experiments; the significance of μ and λ in these new equations being the same as before. Two solutions of this differential equation of general type are

(*a*) $$\eta-\eta_i=\lambda\left(1-e^{-\frac{\mu}{\lambda}t}\right), \qquad \text{when } n=1,$$

and, (*b*) $$\frac{1}{[\lambda-(\eta-\eta_i)]^2}=\frac{2\mu}{\lambda^3}t+\frac{1}{\lambda^2}, \qquad \text{when } n=3.$$

Tests were made with these solutions and it was found that they accorded with the observations only for the first shorter period of gelation, and therefore they may reasonably be rejected in favour of equation (1).

Comparison of the values observed and calculated by equation (1) is to be seen in Tables 1, 2 and 3 which correspond to Figs. 1, 2 and 3 respectively.

It is seen from these tables that equation (1) holds for the first four or five hours of gelation, but that at later periods, the viscosity observed increases more rapidly than is given by this equation. Moreover, as can be seen in Fig. 1, curve 3, a gelation starting as a second case, but very near the critical case, may change gradually into the first case, after a certain lapse of time, so that the curve has an inflexion point. Therefore, the statements given above, although holding exactly for earlier periods of gelation, do not hold for later periods. An equation which holds for the whole course of gelation has not yet been found.

The equation (3) is an equation of the second order reaction with respect to the term $\lambda-(\eta-\eta_i)$. If it is assumed that this term is directly proportional to the concentration of α-glutin, the nature of gelation can be discussed as follows.

From the kinetic theory point of view, the particles of a sol are continuously colliding with each other, and gelation can be regarded as being due to the fact that the particles, or at least a certain proportion of them, unite to form an aggregate particle on coming into contact with each other. In the earlier periods of gelation such a union would occur mainly between two individual particles, the reaction thus being of the second order, as given by equation (3). Later, however, when the number of such aggregate particles becomes larger, the probability of union between an aggregate particle and a single particle, as well as between two aggregate particles, increases, so that the viscosity increases more rapidly than indicated by equation (1). These aggregate particles again unite with others, and become larger and larger

Table 1. *Gelation of* 3 *per cent. gelatin sol at* 18° (*Fig.* 1).
(*Previously heated at* 80°.)

	t (hours)	η	$\eta-\eta_i$ obs.	$\eta-\eta_i$ calc.
				$\mu=3{\cdot}2$, $\lambda=-8{\cdot}6$
No. 1 (Curve 1) Heated for 6 minutes	0	2·15	0	0
	0·1	2·47	0·32	0·33
	0·3	3·22	1·07	1·08
	0·5	4·10	1·95	1·96
	0·7	5·20	3·05	3·03
	1·0	7·28	5·13	5·10
	1·2	9·10	6·95	6·95
	1·4	11·52	9·37	9·37
	1·6	14·75	12·6	12·6
	1·8	19·05	16·9	17·5
	2·05	29·5	27·4	27·7
				$\mu=2{\cdot}2$, $\lambda=-14{\cdot}7$
No. 2 (Curve 2) Heated for 1 hour	0	2·01	0	0
	0·1	2·23	0·22	0·22
	0·2	2·48	0·47	0·45
	0·4	2·96	0·95	0·94
	0·6	3·48	1·47	1·45
	0·8	4·02	2·01	2·00
	1·0	4·58	2·57	2·59
	1·3	5·57	3·56	3·55
	1·7	7·05	5·04	5·02
	2·0	8·25	6·24	6·27
	2·3	9·61	7·60	7·71
	2·8	12·3	10·3	10·4
	3·0	14·0	12·0	12·0
	3·3	15·9	13·9	14·3
	3·55	19·3	17·3	16·6
	4·0	27·7	25·7	21·9
				$\mu=1{\cdot}27$, $\lambda=20{\cdot}0$
No. 3 (Curve 3) Heated for 3 hours	0	1·87	0	0
	0·2	2·12	0·25	0·25
	0·4	2·40	0·53	0·50
	0·6	2·60	0·73	0·73
	0·8	2·84	0·97	0·97
	1·0	3·07	1·20	1·19
	1·3	3·43	1·56	1·53
	1·7	3·83	1·96	1·95
	2·0	4·12	2·25	2·25
	2·5	4·60	2·73	2·74
	3·0	5·06	3·19	3·20
	4·0	5·98	4·11	4·05
	5·0	6·96	5·09	4·82
	6·0	8·09	6·22	5·51
	7·0	9·45	7·58	
	8·0	10·77	8·90	
	9·0	11·96	10·09	
	11·0	14·75	12·88	

	t (hours)	η	$\eta-\eta_i$ obs.	$\eta-\eta_i$ calc.
				$\mu=0{\cdot}80$, $\lambda=2{\cdot}7$
No. 4 (Curve 4) Heated for 6 hours	0	1·75	0	0
	0·1	1·83	0·08	0·08
	0·2	1·90	0·15	0·15
	0·5	2·11	0·36	0·35
	1·0	2·37	0·62	0·62
	1·6	2·62	0·87	0·87
	2·0	2·74	0·99	1·00
	3·0	3·02	1·27	1·27
	4·0	3·32	1·57	1·47
	5·0	3·50	1·75	1·61
	6·0	3·77	2·02	
	7·0	3·98	2·23	
	8·0	4·23	2·48	
	9·0	4·35	2·60	
	11·0	4·84	3·09	
	14·0	5·28	3·53	
	15·0	5·48	3·73	
	17·0	5·90	4·15	
	19·0	6·20	4·45	
	23·0	6·88	5·13	
				$\mu=0{\cdot}45$, $\lambda=0{\cdot}90$
No. 5 (Curve 5) Heated for 11 hours	0	1·66	0	0
	0·1	1·70	0·04	0·04
	0·3	1·78	0·12	0·12
	0·5	1·84	0·18	0·18
	1·0	1·96	0·30	0·30
	2·0	2·11	0·45	0·45
	4·0	2·26	0·60	0·60
	7·0	2·48	0·82	0·70
	9·0	2·56	0·90	
	10·0	2·61	0·95	
	12·0	2·73	1·07	
	14·0	2·83	1·17	
	18·0	2·95	1·29	
	22·0	3·04	1·38	
				$\mu=0{\cdot}16$, $\lambda=0{\cdot}46$
No. 6 (Curve 6) Heated for 20 hours	0	1·57	0	0
	0·2	1·60	0·03	0·03
	0·5	1·64	0·07	0·07
	1·0	1·69	0·12	0·12
	2·0	1·76	0·19	0·19
	3·0	1·80	0·23	0·23
	5·0	1·86	0·29	0·29
	6·0	1·89	0·32	0·31
	9·0	1·94	0·37	0·35
	13·0	1·98	0·41	

Table 2. *Gelation of 3 per cent. gelatin sol at 18° (Fig. 2).*
(Previously heated at 70°.)

	t (hours)	η	$\eta-\eta_i$ obs.	$\eta-\eta_i$ calc.
			$\mu = 3{\cdot}52$, $\lambda = -7{\cdot}9$	
No. 1 (Curve 1) Heated for 6 minutes	0	2·00	0	0
	0·1	2·36	0·36	0·37
	0·3	3·23	1·23	1·22
	0·5	4·27	2·27	2·26
	0·7	5·55	3·55	3·58
	1·0	8·35	6·35	6·35
			$\mu = 2{\cdot}50$, $\lambda = -10{\cdot}4$	
No. 2 (Curve 2) Heated for 2 hours	0	1·93	0	0
	0·1	2·18	0·25	0·26
	0·3	2·76	0·83	0·81
	0·5	3·35	1·42	1·42
	0·7	4·03	2·10	2·10
	1·0	5·18	3·25	3·29
	1·4	7·20	5·27	5·27
	2·0	11·53	9·60	9·61
			$\mu = 1{\cdot}63$, $\lambda = -30{\cdot}0$	
No. 3 (Curve 3) Heated for 6 hours	0	1·84	0	0
	0·1	2·01	0·17	0·16
	0·3	2·33	0·49	0·50
	0·5	2·69	0·85	0·84
	0·7	3·01	1·17	1·19
	1·0	3·56	1·72	1·72
	1·5	4·50	2·66	2·66
	2·0	5·49	3·65	3·66
	2·5	6·58	4·74	4·72
	4·0	11·37	9·53	8·34
			$\mu = 1{\cdot}10$, $\lambda = 4{\cdot}5$	
No. 4 (Curve 4) Heated for 16 hours	0	1·72	0	0
	0·1	1·83	0·11	0·11
	0·3	2·04	0·32	0·31
	0·5	2·21	0·49	0·49
	0·7	2·37	0·65	0·66
	1·0	2·60	0·88	0·88
	2·0	3·20	1·48	1·48
	3·0	3·62	1·90	1·90
	4·0	4·08	2·36	2·22
	5·0	4·48	2·76	
	6·0	4·90	3·18	
	7·0	5·28	3·56	
	9·0	6·08	4·36	
	11·0	6·93	5·21	
	14·0	8·19	6·47	
	16·0	8·98	7·26	
	17·0	9·37	7·65	

	t (hours)	η	$\eta-\eta_i$ obs.	$\eta-\eta_i$ calc.
			$\mu = 1{\cdot}00$, $\lambda = 1{\cdot}95$	
No. 5 (Curve 5) Heated for 20 hours	0	1·70	0	0
	0·1	1·80	0·10	0·095
	0·3	1·96	0·26	0·26
	0·5	2·10	0·40	0·40
	1·0	2·36	0·66	0·66
	2·0	2·69	0·99	0·99
	3·0	3·03	1·33	1·18
	5·0	3·53	1·83	
	8·0	4·19	2·49	
	10·0	4·55	2·85	
	13·0	5·11	3·41	
	15·0	5·48	3·78	
	17·0	5·85	4·15	
	19·0	6·21	4·51	
	25·0	7·20	5·50	
	28·0	7·64	5·94	
			$\mu = 0{\cdot}85$, $\lambda = 1{\cdot}30$	
No. 6 (Curve 6) Heated for 30 hours	0	1·63	0	0
	0·1	1·71	0·08	0·08
	0·3	1·85	0·22	0·21
	0·5	1·95	0·32	0·32
	1·0	2·14	0·51	0·51
	2·0	2·37	0·74	0·74
	3·0	2·53	0·90	0·86
	5·0	2·79	1·16	
	7·0	2·99	1·36	
	9·0	3·21	1·58	
	11·0	3·39	1·76	
	13·0	3·56	1·93	
	17·0	3·83	2·20	
	20·0	4·04	2·41	
	24·0	4·27	2·64	
	32·0	4·72	3·09	
	38·0	5·04	3·41	
	44·0	5·30	3·67	
	48·0	5·50	3·87	
			$\mu = 0{\cdot}83$, $\lambda = 0{\cdot}97$	
No. 7 (Curve 7) Heated for 39 hours	0	1·61	0	0
	0·1	1·69	0·08	0·08
	0·3	1·81	0·20	0·20
	0·5	1·90	0·29	0·29
	1·0	2·05	0·44	0·45
	2·0	2·22	0·61	0·61
	4·0	2·43	0·82	0·75
	8·0	2·76	1·15	
	11·0	2·95	1·34	
	15·0	3·16	1·55	
	23·0	3·47	1·86	
	30·0	3·74	2·13	
	34·0	3·87	2·26	
	39·0	4·02	2·41	
	50·0	4·26	2·65	

Table 3. *Gelation of* 1 *per cent. gelatin sol at* 18° (*Fig.* 3).
(*Previously heated at* 80°.)

	t (hours)	η	$\eta-\eta_i$ obs.	$\eta-\eta_i$ calc.
				$\mu=0{\cdot}21$
				$\lambda=0{\cdot}57$
No. 1 (Curve 1) Heated for 6 minutes	0	1·26	0	0
	0·1	1·28	0·02	0·02
	0·2	1·30	0·04	0·04
	0·5	1·35	0·09	0·09
	0·8	1·39	0·13	0·13
	1·0	1·42	0·16	0·15
	1·5	1·46	0·20	0·20
	2·0	1·50	0·24	0·24
	3·0	1·56	0·30	0·30
	4·0	1·60	0·34	0·34
	5·0	1·64	0·38	0·37
	8·0	1·75	0·49	0·43
	10·0	1·81	0·55	
	13·0	1·90	0·64	
	17·5	2·01	0·75	
	21·0	2·10	0·84	
	26·0	2·20	0·94	
	33·0	2·29	1·03	
	41·0	2·39	1·13	
	47·0	2·45	1·19	
	51·0	2·50	1·24	
	57·0	2·55	1·29	
				$\mu=0{\cdot}17$
				$\lambda=0{\cdot}40$
No. 2 (Curve 2) Heated for 1·2 hours	0	1·244	0	0
	0·1	1·26	0·016	0·016
	0·3	1·29	0·046	0·045
	0·6	1·325	0·08	0·08
	0·8	1·34	0·10	0·10
	1·0	1·36	0·12	0·12
	1·5	1·39	0·15	0·15
	2·0	1·42	0·18	0·18
	3·0	1·46	0·22	0·22
	4·0	1·50	0·26	0·25
	6·0	1·55	0·31	0·29
	9·0	1·62	0·38	0·32
	10·0	1·64	0·40	
	12·0	1·69	0·45	
	16·5	1·77	0·53	
	20·0	1·83	0·59	
	25·0	1·90	0·66	
	32·0	1·98	0·74	
	40·0	2·05	0·81	
	46·0	2·12	0·88	
	50·0	2·16	0·92	
	56·0	2·20	0·96	

	t (hours)	η	$\eta-\eta_i$ obs.	$\eta-\eta_i$ calc.
				$\mu=0{\cdot}10$
				$\lambda=0{\cdot}23$
No. 3 (Curve 3) Heated for 4 hours	0	1·22	0	0
	0·1	1·23	0·01	0·01
	0·3	1·25	0·03	0·03
	0·6	1·27	0·05	0·05
	1·0	1·29	0·07	0·07
	1·5	1·31	0·09	0·09
	2·0	1·33	0·11	0·11
	3·0	1·35	0·13	0·13
	4·0	1·37	0·15	0·15
	6·0	1·41	0·19	0·17
	8·0	1·44	0·22	0·18
	13·0	1·49	0·27	
	17·0	1·55	0·33	
	22·0	1·58	0·36	
	29·0	1·63	0·41	
	37·0	1·67	0·45	
	43·0	1·71	0·49	
	47·0	1·72	0·50	
	52·0	1·74	0·52	
				$\mu=0{\cdot}037$
				$\lambda=0{\cdot}15$
No. 4 (Curve 4) Heated for 11 hours	0	1·20	0	0
	0·3	1·21	0·01	0·01
	0·6	1·22	0·02	0·02
	1·0	1·23	0·03	0·03
	2·0	1·25	0·05	0·05
	3·5	1·27	0·07	0·07
	6·0	1·29	0·09	0·09
	10·0	1·32	0·12	0·11
	15·5	1·35	0·15	0·12
	22·5	1·38	0·18	
	30·0	1·40	0·20	
	40·0	1·42	0·22	

with the lapse of time. They contain, of course, a certain amount of water, gelatin being a hydrophil colloid.

This hypothesis seems very probable, its only weakness being the assumption stated above. It can, indeed, be reasonably regarded that the term $\lambda - (\eta - \eta_i)$ is an increasing function of the concentration of α-glutin. But there is no theoretical or experimental basis for the assumption that the former is directly proportional to the latter.

Therefore the only conclusion which can be definitely deduced from the results of these experiments is that the gelation is, in its early stage at any rate, a second order reaction with respect to the term $\lambda - (\eta - \eta_i)$.

II. On the Hydrolysis of Gelatin by Heating.

Gelatin sol undergoes an irreversible change when it is heated at a high temperature. This change is believed to be a hydrolysis of α-glutin, producing β-glutin, as already mentioned in the foregoing section. Commercial gelatin contains to begin with a certain amount of β-glutin, for heat is applied in its production. Moreover, when it is brought to a sol, heat is again applied, so that a part of the α-glutin changes to β-glutin. Hence the concentration of α-glutin in a gelatin sol of known percentage is quite unknown and varies with the sample used and the conditions of its preparation.

The dynamic study of the hydrolysis of gelatin is merely a study of the variation of the concentration of the α-glutin or its hydrolysis products with the time. However, we have as yet no method of determining the concentration of α-glutin or of β-glutin separately. Moreover, β-glutin may reasonably be regarded, not as a definite substance, but as a mixture of the products at various stages of the hydrolysis caused by heating.

The determination of the viscosity of the sol might be regarded as a method for studying its hydrolysis. But, even when β-glutin is assumed to be a single substance, a gelatin sol is still a mixture of two colloids, namely α-glutin and β-glutin. Now the viscosity of a mixture is not yet known as a definite function of the viscosities and concentrations of its constituents; at least it is not a linear function of the concentrations of the constituents, except in the case of dilute solutions. Therefore the hydrolysis of gelatin cannot be determined by measuring changes in its viscosity. In fact we do not know as yet of any measurable properties of a gelatin sol whose rate of change is proportional to the rate of change of the concentration of α-glutin in the process of its hydrolysis caused by heating.

Now, as a by-product of the experiments of the foregoing chapter, a study was made of the change of viscosity of the sol during the course of its hydrolysis. This study was made, however, for its own intrinsic interest, and not for its bearing on the process of the hydrolysis.

The initial viscosities η_i of the previous chapter are merely the viscosities of a mixture of α-glutin and β-glutin at successive periods of its hydrolysis, measured, however, at a lower temperature, viz. 18°. We shall, in what

follows, represent the time of heating by τ, to distinguish it from the time t in the course of subsequent gelation.

Von Schröder [1903] stated that the change in the viscosity of the hydrolysing gelatin sol, which he measured at 25°, was represented by an equation of the first order reaction, viz.:

$$-\frac{d\eta_i}{d\tau} = k_1(\eta_i - \eta_{i\infty}),$$

i.e.

$$k_1 = \frac{1}{\tau} ln \frac{\eta_{i0} - \eta_{i\infty}}{\eta_i - \eta_{i\infty}},$$

where η_{i0} and $\eta_{i\infty}$ are respectively the values of η_i at the beginning of the heating and after a long period of heating, while k_1 is a constant usually called the velocity coefficient.

Levites [1908], on the other hand, stated that this equation did not hold for his experiments with gelatin, agar and the sodium salt of α-thymonucleic acid. This equation fails, too, to represent correctly the results of the present experiments, whereas the equation of the third order reaction

$$\left.\begin{aligned} -\frac{d\eta_i}{d\tau} &= k_3(\eta_i - \eta_{i\infty})^3 \\ \textit{i.e.}\quad k_3 &= \frac{1}{2\tau}\left[\frac{1}{(\eta_i - \eta_{i\infty})^2} - \frac{1}{(\eta_{i0} - \eta_{i\infty})^2}\right] \\ \text{or}\quad k_3 &= \frac{1}{2(\tau_1 - \tau_2)}\left[\frac{1}{(\eta_{i1} - \eta_{i\infty})^2} - \frac{1}{(\eta_{i2} - \eta_{i\infty})^2}\right] \end{aligned}\right\} \quad \ldots\ldots\ldots\ldots\ldots\ldots(4)$$

where k_3 is a velocity coefficient varying with the temperature of heating and the concentration of α-glutin is found to be satisfactory. This can be seen from Tables 4, 5 and 6, in which the η_i (calc.) column is calculated from equation (4) using a mean value of k_3, and k_2 is the velocity coefficient corresponding to the equation of the second order reaction, *i.e.*

$$k_2 = \frac{1}{\tau_1 - \tau_2}\left[\frac{1}{\eta_{i1} - \eta_{i\infty}} - \frac{1}{\eta_{i2} - \eta_{i\infty}}\right].$$

Table 4. *Hydrolysis of 3 per cent. gelatin sol heated at 80°.*
(Data of η_i taken from Table 1.)

τ (hours)	η_i	η_i (calc.)	k_3	k_2	$\cdot 434k_1$
0·1	2·15	—	—	—	—
1·0	2·01	2·02	0·33	0·26	0·088
3·0	1·87	1·86	0·29	0·20	0·060
6·0	1·75	1·75	0·30	0·18	0·047
11·0	1·66	1·65	0·29	0·15	0·034
20·0	1·57	1·57	0·31	0·13	0·025
∞	1·30	—	—	—	—
		Mean	0·304		

Table 5. *Hydrolysis of 3 per cent. gelatin sol heated at 70°.*
(Data of η_i taken from Table 2.)

τ (hours)	η_i	η_i (calc.)	k_3	k_2	$\cdot 434k_1$
0·1	2·00	—	—	—	—
2·0	1·93	1·935	0·126	0·083	0·024
6·0	1·84	1·843	0·118	0·072	0·019
16·0	1·72	1·719	0·114	0·060	0·014
20·0	1·70	1·689	0·106	0·054	0·012
30·0	1·63	1·635	0·119	0·053	0·011
39·0	1·61	1·602	0·108	0·046	0·009
∞	1·30	—	—	—	—
		Mean	0·115		

Table 6. *Hydrolysis of* 1 *per cent. gelatin sol heated at* 80°.
(*Data of* η_i *taken from Table* 3.)

τ (hours)	η_i	η_i (calc.)	k_2	k_3	$\cdot 434k_1$
0·1	1·26	—	—	—	—
1·2	1·244	1·244	10·4	1·16	0·057
4·0	1·22	1·221	11·1	1·07	0·045
11·0	1·20	1·198	9·6	0·76	0·028
∞	1·14	—	—	—	—
		Mean	10·4		

Conclusions.

1. The change of viscosity η of gelatin sol with respect to time t in the early stages of its gelation is represented by the equation

$$-\frac{d}{dt}[\lambda-(\eta-\eta_i)]=\frac{\mu}{\lambda^2}[\lambda-(\eta-\eta_i)]^2,$$

i.e.

$$\eta-\eta_i=\frac{\mu t}{1+\frac{\mu}{\lambda}t},$$

μ being the rate of increase of viscosity at the initial moment of gelation, increasing with the initial concentration of the α-glutin. The constant λ is also larger, the larger the initial concentration of α-glutin, being positive or negative according as the initial concentration is below or above a certain critical concentration. When positive, λ is the asymptotic value of the viscosity attained after a long time. When λ is negative, gelation reaches its final stage after a finite time given by $t=-\frac{\lambda}{\mu}$.

The bearing of the above equation on the nature of gelation is discussed at some length.

2. The change of viscosity η_i of gelatin sol with respect to time τ in the course of its hydrolysis caused by heating is found to be represented by the equation,

$$-\frac{d\eta_i}{d\tau}=k(\eta_i-\eta_{i\infty})^3,$$

i.e.

$$2k\tau=\frac{1}{(\eta_i-\eta_{i\infty})^2}-\frac{1}{(\eta_{i0}-\eta_{i\infty})^2}.$$

The writer wishes to express his thanks to Dr Yoshio Ishida for some valuable suggestions which assisted him in finding the empirical gelation-formula adopted. Further studies on the process of gelation with respect to concentration and temperature will be reported in a following paper.

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XXV. NITROGEN PARTITION IN THE URINE OF THE RACES IN SINGAPORE.

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Introduction.

The observations here recorded form the continuation of a research directed mainly to the question of the nitrogen partition in the urine of individuals belonging to different races, including a Brahmin, a Chinese, a Tamil, a European, a Malay, a Hindoo, a Eurasian, a Bengali and a Sikh. The European has been a lecturer for more than five years and the other subjects have studied medicine for at least two years at the local school.

The first five of these subjects have been under observation since October 1916. Some of the results dealing with their diet, nutrition and excretion have been published [1917] and where comparable they support the evidence of the present work.

Previous Research.

A good deal of research has been published connected with diet and nutrition in various tropical climates but scarcely any dealing in detail with the nitrogen partition, the only searching work being that carried out by Young [1915], who studied the protein metabolism of white races living in tropical Queensland. His results exhibit no marked variations from the averages obtained in temperate climates.

Methods.

In all cases analyses of the urine were made for seven—in most cases consecutive—days. Care was taken to prevent decomposition of the specimens by the addition of 2 cc. of 5 per cent. solution of thymol in chloroform.

Kjeldahl's method was employed for estimation of the total nitrogen, Benedict's method for urea, Malfatti's method for ammonia, Johnson's method for creatinine, Salkowski's method for purine bases, Ludwig-Salkowski's method for uric acid, Folin's method for inorganic and ethereal sulphates, Volhard-Arnold's method for chlorides, the uranium acetate method for phosphates, and Folin's method for the total acidity.

Table I. *Statistics regarding Subjects under Observation.*

Subject	Race	Birthplace	Age in years	Height in inches	Weight in kilos.	Years resident in Malaya	Years resident in Singapore
Brahmin	Tamil	Ceylon	23	63	46·1	4·0	3·5
Chinese	Chinese	Singapore	22	62	41·3	22·0	22·0
Tamil	Tamil	Ceylon	28	62	61·7	9·0	4·0
European	British	Australia	35	67	55·6	5·0	5·0
Hindoo	Tamil	Ceylon	23	62	46·9	6·5	2·5
Malay	Malay	Malaya	20	63	56·5	20·0	6·0
Eurasian	Eurasian	Malaya	20	61	49·5	20·0	2·0
Bengali	Bengali	Bengal	18	68	59·1	17·0	3·0
Sikh	Sikh	Punjab	23	67	48·2	12·0	3·0

The composition of the foods and their heat values were taken from a standard work [Leach 1911].

Statistics regarding the subjects are given in Table I. Table II shows the average figures for the nitrogen partition, and Table III the average figures for the non-nitrogenous excretion.

Present Observations.

Brahmin. This man was a vegetarian, his diet consisting of bread, butter, fruit, vegetables, rice (the staple), milk, jam, pulses, tea, coffee, cocoa, sugar, salt and spices. He was under observation for three years, and although his diet varied considerably in quantity, it did not vary in quality. The daily total nitrogen output for three years varied from 4·3 g. to 8 g. He lost 4 lbs. weight in three years.

He lived in the school hostel at the time the observations here recorded were made, his average daily diet containing protein 54 g., fat 37, carbohydrate 288, the heat value being 1746 kilocalories, and the daily total nitrogen output averaging 5·1 g. (Table II). The ammonia nitrogen and creatinine nitrogen were not so reduced in quantity as the total nitrogen, urea nitrogen and uric acid nitrogen, when compared with the standard for Europe which is shown in Table II. Therefore the percentages of ammonia nitrogen and creatinine nitrogen were higher than the standard percentages, whilst the percentages of urea nitrogen and uric acid nitrogen were not so greatly altered.

Hindoo. This man partook of the same diet as the Brahmin. He belonged to the same race—Tamil. His average daily diet during the week of observation contained protein 63 g., fat 32, carbohydrate 354, the heat value being 2007 kilocalories. His figures for nitrogen partition, on the whole, confirmed the results for the Brahmin. The average daily total nitrogen output was 5·97 g. representing 37·3 g. protein. The ammonia nitrogen and the creatinine nitrogen suffered a smaller reduction in quantity than the other nitrogenous excretions and were increased in percentage when compared with the standard for Europe (Table II).

Chinese. This student was under observation for three years. His diet

was mixed, containing rice (the staple), bread, butter, milk, jam, beef, mutton, pork, chicken, fish, eggs, vegetables, fruit, sugar, salt, tea, coffee and spices. During six months of observation his daily diet averaged protein 60 g., fat 43, carbohydrate 227, the heat value being 1577 kilocalories and the average daily nitrogen excretion 9·25 g. [Campbell 1917]. He gained 3 lbs. weight in three years.

For the week in which the present observations were made the average daily nitrogen output was 8·60 g. (Table II). When compared with the standard for Europe, the nitrogen excretions, with the exception of the ammonia nitrogen and creatinine nitrogen, were much reduced in quantity, so that the percentages of ammonia nitrogen and creatinine nitrogen were higher than the standard for Europe.

European. This lecturer was under observation for five years in Singapore. During the first three years he lost 14 lbs. in weight; during the last two years his weight was fairly constant. With the loss of weight there was a fall in the diet. The nitrogen output fell from 15 g. to 12 g. per diem. He used an ordinary mixed diet, practically identical in type with the diet in Europe.

The average daily diet during the week of observation consisted of protein 80 g., fat 59, carbohydrates 216, the heat value being 1763 kilocalories, and the average nitrogen excretion 11·70 g. (Table II), which accounted for 73·1 g. protein. The ammonia nitrogen, 0·69 g., was higher than the standard. All the other nitrogenous substances were excreted in smaller quantities than the standard for Europe. The creatinine nitrogen 0·47 g. was not greatly reduced in amount.

Malay, Eurasian, Tamil, Bengali. These four students are considered together because they sat at the same table in the school hostel, and used the same mixed diet, resembling in substance that taken by the Chinese student. The Malay and the Tamil had been under observation since 1916. On the hostel diet the Tamil lost 1 lb. and the Malay gained 5 lbs. in weight in three years. The Tamil's daily diet for three months averaged protein 58 g., fat 32, carbohydrate 277, the heat value being 1672 kilocalories and the total nitrogen excretion 8·24 g. per diem. The Malay's diet was similar, averaging protein 57 g., fat 31, carbohydrate 239 per diem [Campbell 1917].

In the present series of analyses the average daily nitrogen output was 8·74 g. for the Malay, 9·34 for the Tamil, 9·89 for the Eurasian and 7·57 for the Bengali (Table II). In all cases the nitrogenous excretions were much smaller in amount than those of the standard for Europe, with the exception of the ammonia and creatinine. In all subjects the ammonia nitrogen was excreted in greater amount and the creatinine nitrogen in somewhat smaller amount than the standard.

Sikh. This student used a mixed diet similar to that taken by the four students mentioned above, but he lived outside the school hostel. His average daily nitrogen output was 8·64 g. (Table II), the nitrogen partition resembling that of the Malay.

Table II. *Nitrogen Partition.* (Average figures.)

Subject	Diet	Total N	Urea N		Ammonia N		Creatinine N			Uric acid N		Purine bases N		Rest	Total N per kilo of body weight
		g.	g.	%	g.	%	g.	%	coeff.	g.	%	g.	%	%	g.
Brahmin	Vegetable	5·10	3·90	76·59	0·50	9·88	0·40	7·99	8·8	0·08	1·63	0·013	0·25	3·66	0·110
Chinese	Mixed	8·60	7·02	81·63	0·54	6·28	0·36	4·26	8·7	0·14	1·63	0·008	0·09	6·11	0·208
Tamil	,,	9·34	7·67	82·09	0·62	6·72	0·42	4·52	6·8	0·16	1·75	0·015	0·16	4·76	0·151
European	,,	11·70	9·78	83·59	0·69	5·96	0·47	4·05	8·5	0·16	1·37	0·016	0·14	4·89	0·210
Hindoo	Vegetable	5·97	4·75	79·60	0·39	6·60	0·38	6·36	8·1	0·09	1·62	0·009	0·15	5·67	0·127
Malay	Mixed	8·74	6·89	78·91	0·69	7·91	0·44	5·08	7·8	0·17	1·91	0·013	0·14	6·05	0·154
Eurasian	,,	9·89	7·98	80·75	0·58	5·86	0·50	5·06	10·1	0·14	1·45	0·009	0·09	6·79	0·199
Bengali	,,	7·57	6·08	80·37	0·63	8·33	0·45	5·92	7·6	0·13	1·76	0·017	0·22	3·40	0·128
Sikh	,,	8·64	7·03	81·45	0·67	7·76	0·43	4·97	8·9	0·11	1·31	0·021	0·24	4·27	0·179
Standard for Europe	,,	16·00	13·60	85·00	0·53	3·30	0·57	3·60	7 to 11	0·25	1·56	0·025	0·15	6·39	0·200

Conclusions.

On the various diets, vegetarian and mixed, used by the Singapore subjects, the nitrogen partition differed from the standard for Europe in the high percentages of ammonia nitrogen and creatinine nitrogen. As regards creatinine, the percentage was high because creatinine, being mainly of endogenous origin, is not greatly reduced in absolute quantity when the total nitrogen is reduced. It will be shown that, in most cases, the amount of ammonia nitrogen was excessive.

Race had no influence on the nitrogen partition, the figures for the different races resembling one another fairly closely, after making due allowance for differences in diets.

Nitrogenous Excretions.

Total Nitrogen. In all cases the daily total nitrogen was much lower than that of the standard for Europe; but if we consider the total nitrogen per kilogram of body weight it will be observed that the figures 0·210 g. for the European, 0·208 for the Chinese, 0·199 for the Eurasian, resembled the standard 0·200 (Table II). The figures for the Brahmin 0·110 and for the Hindoo 0·127 were the lowest mainly because the vegetable protein eaten by them was not so easily digested as the flesh protein taken by the other subjects. The vegetarians used quite as much protein in their food as many of the other subjects. Most of the subjects used about half the standard quantity of protein. McCay, Chittenden and others have already pointed out that it is possible to maintain protein equilibrium on less than half the standard for Europe. The Brahmin and the Hindoo, who had the two lowest figures, were the best students of their years. However, the average Singapore student under observation had not the physique and energy of the average student in Europe nor of the average European resident in Singapore. This supports McCay's views [1910], that races who live on diets of high protein content have better physique and energy than those whose diet contains less protein.

Urea Nitrogen. The text-books state that the urea nitrogen, 16 g., amounts to about 85 per cent. of the total nitrogen excreted, both varying directly with the amount of protein absorbed. According to Folin, when the total nitrogen output falls to 7—8 g., the urea nitrogen is reduced to about 79 per cent. My observations are in general agreement with these conclusions.

Ammonia Nitrogen. In all cases the percentages of ammonia nitrogen were higher than the standard, in some cases very much higher. At first sight this might be expected, as Folin found that the ammonia nitrogen forms about 5 per cent. of the total nitrogen, if the total nitrogen is only 7 or 8 g. Moreover, Malfatti's method of ammonia estimation, which I employed, gives higher results than Folin's method because the former includes also the ammonia of any amino-acids in the urine. Making liberal allowance for the

ammonia of the amino-acids, the ammonia nitrogen may form 6 per cent. of the total nitrogen, if the latter is only 7 or 8 g. and if Malfatti's method is used. But in many cases my percentages were much higher than this; and the absolute quantities of ammonia were also higher than the standard, except in the two vegetarians, Brahmin and Hindoo (Table II); even in them the amounts were higher than one would reasonably expect on their diet. In my previous researches on Singapore medical students [1917] and on Singapore labourers [1918] large amounts of ammonia were detected on many occasions.

Normally the absolute amounts of urea and ammonia rise and fall together with the total nitrogen. In most of my subjects there was a fall in the amounts of total nitrogen and urea nitrogen but a rise in the ammonia nitrogen, which condition indicates an acidosis. What was the cause? Taking all the diets into consideration there was nothing in the food to account for the increase of ammonia. Again the analyst's reports showed that the Singapore water was particularly pure. It is held that the sole use of the urinary ammonia is for neutralisation of acids formed during metabolism. Probably in the Singapore subjects there was a disturbance in metabolism. The severe climatic conditions in Singapore may be responsible, seeing that so many different subjects were affected. Singapore climate is noted for the lassitude it produces. Eight months change in a temperate climate is taken every four years, a more frequent change being usual for women. Further experiments are required to determine whether the acidosis explains any of the discomforts and ill-effects of the climate. Young [1915] working in Townsville, Queensland, has not found any increase in the ammonia nitrogen, but he has noted an increase in the neutral sulphur, which he suggests may be due to an increased tissue metabolism. Townsville and Singapore climates are not very much alike. Singapore, which is much nearer the equator, does not enjoy a cool season, the temperature averaging 80° F. for the whole year; nor does the Townsville climate affect the health of Europeans in the same degree as that of Singapore.

It is well known that fever—that is, a rise of body temperature—causes an increase in urinary ammonia. Hot, moist and still air in Singapore produces great discomfort and a rise of body temperature [Campbell 1919]. In the cotton sheds in England a rise of mouth temperature is observed when the wet bulb thermometer exceeds 75° F. [quoted by O'Connell 1913]. In Singapore this wet bulb reading is often exceeded for weeks at a time. Probably this caused the acidosis observed by me.

Creatinine Nitrogen. The creatinine coefficients (that is milligrams of creatinine per kilogram of body weight) of my series resembled the standard coefficient for Europe, which ranges from 7—11. My figures varied from 6·8 for the Tamil to 10·1 for the Eurasian (Table II). The Brahmin and the Hindoo, on a diet containing neither creatine nor creatinine, had practically the same coefficient as the subjects on a diet containing these substances.

Obviously all the creatinine of the former subjects was endogenous. The percentages of creatinine were higher than the standard because creatinine, being mainly of endogenous origin, is not lowered in quantity when the total nitrogen is decreased.

Uric Acid Nitrogen. The quantities obtained were all lower than the standard figures, but the percentages agreed with the standard. This was to be expected since uric acid is decreased by cutting down the protein (nucleo-protein) of the diet. The decrease in quantity was particularly noticeable in the case of the Brahmin and the Hindoo, who used a vegetable diet. Most of their uric acid was evidently endogenous.

Purine Nitrogen. None of the subjects excreted so large a quantity as the standard, because they did not consume so large a quantity of the purine bases contained in tea and coffee as the average man in Europe. The tea taken by the Singapore European was a very weak infusion, and he never drank coffee.

Non-Nitrogenous Excretions.

Phosphates. The figures varied from 1·6 g. for the European to 0·9 g. for the Bengali (Table III). The ratio of phosphoric oxide to nitrogen is given in text-books as 1 to 5 or 6; that is about 2·5 g. P_2O_5. With the exception of the Brahmin and the Hindoo where the ratio was 1 to 5, the average ratio in my results was 1 to 7·5. The Singapore diets evidently contained smaller quantities of absorbable phosphate than the standard diet for Europe.

Table III. *Non-nitrogenous excretion.* (Average figures.)

Subject	Volume in cc.	Specific gravity	Acidity in cc. *N*/10 NaOH	P_2O_5	NaCl	Inorganic SO_3 g.	Ethereal SO_3 g.
Brahmin	1207	1·013	194	1·1	8·9	0·541	0·097
Chinese	1113	1·014	285	1·3	7·5	1·250	0·107
Tamil	997	1·017	348	1·1	4·9	1·174	0·117
European	1102	1·017	375	1·6	5·4	1·416	0·108
Hindoo	1016	1·020	262	1·1	8·5	0·736	0·147
Malay	1006	1·017	260	1·3	7·0	0·957	0·129
Eurasian	1056	1·017	286	1·3	5·7	1·174	0·085
Bengali	1328	1·009	188	0·9	3·2	0·829	0·102
Sikh	593	1·024	332	1·3	3·0	1·056	0·092

Sulphates. Only the inorganic and ethereal sulphates were estimated owing to the failure to obtain the necessary reagents, etc., for the estimations of the neutral and total sulphur.

The inorganic sulphates weigh about 2·5 g. in the standard for Europe and arise chiefly from the oxidation of the sulphur of protein. In my observations the amounts of inorganic sulphate followed closely the variations of the total nitrogen, the average figures ranging from 0·541 g. for the Brahmin to 1·416 for the European, the total nitrogen figures being 5·1 and 11·7 respectively.

The ethereal sulphates, which have their origin mainly in putrefaction taking place in the intestine, varied considerably in my observations. When present in relatively large amounts, constipation was present and a purgative readily reduced the quantity. High proportions were observed in the vegetarians, the Brahmin and the Hindoo.

Chlorides. The standard figure for Europe is 11 g. In my series the average amounts varied from 3 g. for the Sikh to 8·9 for the Brahmin. The latter and the Hindoo excreted the greatest quantities, probably owing to the large amounts of potassium salts in their diet. Bunge has shown that potassium salts deprive the blood of its chloride, whence the craving for more on a vegetable diet [quoted by Howell 1915].

It is well known that individuals have been kept in good condition on 1 or 2 g. per diem with experimental diets, so that the Sikh's figure was not very low.

Acidity. Calculated in cc. *N*/10 NaOH, the acidity varied from 188 in the Bengali to 375 in the European; or, calculated as HCl, it varied from 0·68 to 1·36 g. The standard figures are given in text-books as 1·5 to 2·3 g. HCl. One would expect the acidity to fall in the Singapore subjects because their diet contained less protein than the standard; and abundant perspiration reduces acidity.

Summary.

1. The manner in which the nitrogen is distributed in the urine of a Brahmin, a Chinese, a Tamil, a European, a Hindoo, a Malay, a Eurasian, a Bengali and a Sikh, all attending the Singapore Medical School, is considered.

2. The absolute quantities of total nitrogen, of urea nitrogen, and of uric acid nitrogen, are much lower than the standard figures for Europe, but the percentages of urea nitrogen and uric acid nitrogen do not differ materially from the standard. In the Chinese, the European and the Eurasian the total nitrogen per kilogram of body weight is about the same as the standard; in the other subjects the figures are lower than the standard.

3. The absolute quantity of purine nitrogen is much lower in all subjects except the Sikh, in whom it is slightly lower than the standard. The percentages vary greatly.

4. In all cases the percentage of ammonia nitrogen is increased. In many cases the absolute amount is increased, and in some cases the increase is well marked. This confirms observations on Singapore students and labourers, already published. The diet and the water are not responsible. It is considered that the excess is due to an increase of acid substances in the blood, owing to a disturbance in metabolism, and that the climate of Singapore may be responsible, since so many different subjects, living under such different circumstances and partaking of different diets, are similarly affected.

5. The quantity of creatinine nitrogen is slightly lower than the standard for Europe but the percentage is higher—in some cases much higher—because

the reduction in the creatinine nitrogen is much less than the reduction in the total nitrogen. This supports the theory that creatinine is mainly of endogenous origin and that it is not lowered greatly by reducing the protein intake. The creatinine coefficient for Singapore resembles very closely that given for Europe.

6. Race apart from diet has no influence on nitrogen partition.

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XXVI. CHEMICAL STRUCTURE AND ANTIGENIC SPECIFICITY. A COMPARISON OF THE CRYSTALLINE EGG-ALBUMINS OF THE HEN AND THE DUCK.

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Any rational theory of immunity involves a conception of the relation between an antigen and its corresponding antibody. The side-chain theory of Ehrlich, to which immunological investigation in the past two decades has largely owed its direction, gave a purely chemical conception of this relation. More recently attempts have been made to explain certain immunity reactions, such as specific precipitation and agglutination, on purely physical lines, as the mutual precipitation of oppositely charged colloids. This latter hypothesis, which would make the properties of an immune serum depend upon the electric charge on its proteins, and enable these to react with any oppositely charged amphoteric colloid, clearly provides no explanation for the specific discrimination which is the characteristic of immunity reactions. There is evidence, on the other hand, to show that, whatever be the exact nature of the relation between antigen and antibody, it is dependent on the stereochemical structure of the molecule of the antigen. Ten Broek [1914] showed that a racemised protein, which Dakin and Dudley [1913] had found to be quite unaffected by proteolytic enzymes, had also lost all power of evoking the production of antibodies. It was immunologically an inert substance.

This observation suggests a possibility of accounting for the hitherto puzzling phenomenon of the antigenic disparity between proteins, which to ordinary methods of physical and chemical investigation seem to be identical. Corresponding proteins from different species often yield, to an ordinary hydrolytic analysis, the same amino-acids in apparently identical proportions; yet an immunity reaction, such as specific precipitation or anaphylaxis, will discriminate clearly between them. Though the amino-acids are the same, however, and present in identical proportions, it is evident that change in the order of their linkage in the molecule makes possible an immense number of variations in its structure. That specific differences, between corresponding proteins from allied species, might be due to such differences in the intimate pattern of the molecule, was indicated by some recent results.

When a protein is racemised as far as possible by Kossel's method, and subsequently subjected to acid hydrolysis, it is found that certain of the amino-acids have partly or wholly retained their natural optical activity. Dakin [1912] gave reason for believing that the amino-acids thus escaping racemisation are those occupying the terminal positions in the peptide chains, of which the protein molecule is built. By this method Dudley and Woodman [1915] were able to obtain a first indication of structural difference between the caseinogen of cow's and sheep's milk. There was clearly a possibility that this type of structural difference, of which the method only gives a partial indication, might account for the existence of antigenic distinction in cases where an explanation was hitherto wanting.

It seemed to be worth while, therefore, to take two such pure similar proteins, with good antigenic properties, and to examine, on the one hand, their structure, with the help of the racemisation method, and to test, on the other hand, the possibility of distinguishing between them by the highly specific anaphylactic reaction. In the case of the proteins we chose for this purpose the crystalline albumins from the eggs of the domestic fowl and the duck. Preliminary experiments, recorded by Dale and Hartley [1916], had given reason to expect that no difference would be detected by the anaphylactic reaction. If this had been confirmed, and if evidence of structural difference had been obtained, the possibility of explaining antigenic specificity along these lines would have been seriously weakened. The outcome of our experiments, however, has been to give definite evidence of difference in the arrangement of the amino-acids, and, with a more careful and extended series of experiments than those of Dale and Hartley [1916], a clear discrimination between the two proteins by the anaphylactic reaction.

The preparation of the pure, crystalline albumins, and the comparison by the chemical method, were carried out by Dakin, whose share in the work was completed many months ago, before his recently published method for separation of amino-acids was available. Samples of the albumins were sent at the same time to Dale, who was prevented, however, by pressure of other work, from undertaking until recently the comparison by means of the anaphylactic reaction, for which he is responsible. It was thought better to hold back the chemical findings until the biological results were available.

Chemical Examination.

The albumins were prepared from the whites of hens' and ducks' eggs of unquestionable origin. The method employed for the crystallisation of the albumins was that described by Hopkins [1901] and the products were twice crystallised. In the case of the duck albumin the initial crystallisation was induced by adding rather more acetic acid than that necessary for the crystallisation of hen albumin. Rather over 300 g. of each of the purified proteins were dissolved in water and then dialysed so as to remove most of the adhering

ammonium sulphate. The resulting solutions containing 8—10 per cent. of protein were incubated for 23 days at 37° with the addition of the requisite quantity of caustic soda to bring the concentration of alkali up to half normal. At the end of the incubation period, the alkaline mixtures were hydrolysed by boiling with sulphuric acid and the resulting amino-acids were separated according to the usual methods previously employed in experiments on the racemisation of proteins.

The results as to the relative quantitative yields of individual amino-acids from the two proteins are not considered sufficiently accurate for reproduction, but in general it may be said that they appeared remarkably similar, especially as regards the high yield of phenylalanine and arginine, and relatively low yield of histidine, aspartic acid and leucine.

The results follow:

Amino acid	"Racemised" hen albumin	"Racemised" duck albumin	Comments
Alanine (1)	Not racemised	Not racemised	No difference
Valine (2)	Partly racemised	Partly racemised	,, ,,
Leucine (3)	Mostly racemised	Mostly active	*A definite difference*
Proline (4)	,, ,,	Mostly racemised	No difference
Phenylalanine	Completely inactive	Completely inactive	,, ,,
Tyrosine (5)	Inactive	Inactive	,, ,,
Aspartic acid (6)	Mostly inactive, some active	Completely inactive	*Definite difference*
Glutamic acid	Completely inactive	,, ,,	No difference
Histidine (7)	,, ,,	Mostly active	*Definite difference*
Arginine	Active	Active	No difference
Lysine (8)	Inactive	Inactive	,, ,,

(1) The specific rotations for the alanine hydrochlorides were + 6° and + 7·5° respectively compared with a theoretical value of + 10° but this represents no greater racemisation than usually occurs in the separation of the active alanine from proteins. No racemisation of the alanine group in the proteins by the alkali is indicated.

(2) The valine fraction was not obtained perfectly free from leucine and in both cases was mostly racemised.

(3) A marked difference was noted here. The first large fraction of pure leucine (9 g.) crystals was absolutely inactive in the case of the hen albumin while the corresponding fraction (7 g.) from the duck albumin was almost exclusively the *l* form, $[\alpha]_D = + 15°$ in 20 per cent. hydrochloric acid. Theoretical value = + 15·6°. The whole of the leucine from the duck albumin had a mean rotation in hydrochloric acid of + 13·3° indicating about 85 per cent. of the active variety, while the corresponding rotation of the hen albumin leucine was + 4·3°, and this is probably partly due to contaminating valine. Certainly not more than 27 per cent. of the leucine from the racemised hen albumin was in the active form.

(4) Most of the proline from both preparations was inactive. The hen product had a rotation in water of − 5° while the proline from duck egg

albumin showed a value of − 9·9°. Pure *l*-proline has a specific rotation in water of − 77·4°, and while much racemisation ordinarily occurs in the process of isolation, the above values are much lower than those previously observed even from other racemised proteins, so that it appears that most of the proline groups in both proteins were inactivated by the action of alkali.

(5) No perfectly pure specimens of tyrosine were obtained, since the more soluble inactive acid in small amounts is hard to separate from leucine and other impurities. No laevo-rotation was observed in any case.

(6) The whole of the aspartic acid from the duck egg albumin was optically inactive as well as the mother-liquors from which aspartic acid was separated, so that it is certain that none of the active acid was present. From the hen egg albumin, in addition to a considerable amount of racemic aspartic acid, a small amount (0·3 g.) of the pure laevo-acid was separated. It decomposed around 270°, was faintly laevo-rotatory in alkaline solution, and had $[\alpha]_D^{20^\circ} = + 24{\cdot}5^\circ$ in hydrochloric acid solution (3 mols). Titration with standard alkali using litmus as indicator proved the purity of the product and freedom from leucine. Optically pure aspartic acid under similar conditions shows a specific rotation of + 25·7°. Since in any case *l*-aspartic acid is largely racemised during its separation by the ester method, the detection of a little of the laevo-acid must be taken to indicate the original presence of considerably more of the active acid.

(7) The whole of the histidine fraction from the hen egg albumin was completely inactive both as free base and as salts. On the other hand the histidine from the duck egg albumin, though small in quantity, was strongly laevo-rotatory, having $[\alpha]_D^{20^\circ} = - 27{\cdot}2^\circ$, compared with − 39° for the optically pure base. 70 per cent. of the histidine was therefore made up of the laevo-variety. In each case the histidine was successively separated and identified as phosphotungstate, silver salt, precipitation with mercuric sulphate, and picrolonate.

(8) In neither case could any dextro-lysine be detected, but it is noteworthy that in each case the lysine fraction was feebly laevo-rotatory. The cause of this is obscure, but it was certainly not due to contamination with proline.

Anaphylactic Reactions.

The experiments were all made on guinea-pigs, each of which received a preparatory injection of 1 mg. of one or other of the albumins. After intervals varying from 19 to 31 days they were tested for sensitiveness. Most of the tests were made, by the now familiar method, on the isolated uterine muscle, young virgin females being chosen for this purpose. The use of this method enabled sensitiveness to both albumins to be tested on the same animal. In most cases some degree of sensitiveness to both was present. In many a high degree of sensitiveness to the albumin not used for the preparatory injection was observed. In all cases, however, a preferential sensitisation

to the antigen given in the preliminary injection could be detected. Even when a small first dose of the non-specific antigen caused an apparently maximal contraction, the uterine muscle could be completely desensitised to this and yet retain sensitiveness to the specific one; whereas desensitisation to the specific antigen left the muscle completely insensitive to the other. In some cases the specificity was apparently absolute, the muscle being indifferent to a large dose of the non-specific and subsequently reacting typically with the specific antigen.

A small confirmatory series of experiments was made on intact animals, the doses being injected into the jugular vein.

The albumins were always dissolved in physiological saline solution, and the greatest care was exercised, by the use of separate sets of measures, pipettes, etc., to avoid any possibility of contaminating one solution with traces of the other.

Experiments on the Isolated Uterus.

The following are characteristic records.

Expt. 1. Sensitised with 1 mg. of duck albumin. 19th day.

1*st horn.*	(1) 0·1 mg. Hen. No reaction.
	Change Ringer's solution.
	(2) 0·1 mg. Duck. Good but not maximal contraction.
	Change Ringer.
	(3) 1 mg. Hen. Small reaction.
	Change Ringer.
	(4) 1 mg. Hen. Nil. (Desensitised to hen.)
	(5) 1 mg. Duck. Good but not maximal reaction.
2*nd horn.*	Not tested.

Expt. 2. Sensitised with 1 mg. hen albumin. 21st day.

1*st horn.*	(1) 0·1 mg. Duck. Nil.
	(2) 0·1 mg. Hen. Maximal contraction.
	Change Ringer.
	(3) 0·1 mg. Hen. Nil.
2*nd horn* (see Fig. 1).	(1) 2 mg. Duck. Nil.
	(2) 0·1 mg. Hen. Maximal contraction.
	Change Ringer.
	(3) 0·1 mg. Hen. Nil.
	(4) 1 mg. Hen. Nil.

This is an example of very perfect specificity.

Expt. 3. Sensitised with 1 mg. hen. 30th day.

1*st horn.*	(1) 0·1 mg. Duck. Maximal contraction.
	Change Ringer.
	(2) 0·01 mg. Hen. Weak contraction.
	(3) 0·01 mg. Hen. Nil.
	(4) 0·1 mg. Hen. Moderate contraction.
	(5) 1 mg. Hen. Very weak contraction.

2nd horn.

(1) 0·01 mg. Duck. Moderate contraction.
(2) 0·1 mg. Duck. Nil.
(3) 1 mg. Duck. Weak contraction.
Change.
(4) 1 mg. Duck. Nil. (Desensitised to duck.)
(5) 0·01 mg. Hen. Weak contraction.
(6) 0·1 mg. Hen. Moderate contraction.
Change Ringer.
(7) 1 mg. Duck. Nil.
Change Ringer.
(8) 1 mg. Hen. Moderate contraction.

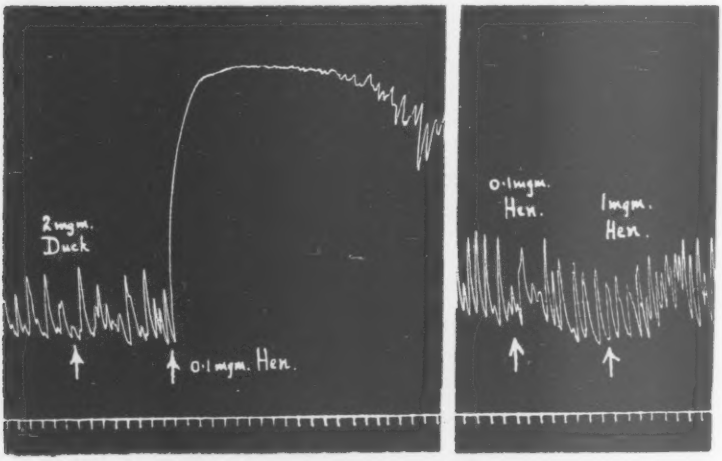

Fig. 1. See Expt. 2, 2nd horn. Sensitised with 1 mg. of Hen albumin, 21st day.

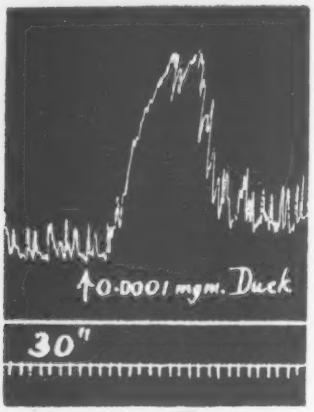

Fig. 2. See Expt. 4, 2nd horn. Effect of 1 part of specific antigen (Duck albumin) in 500 millions of Ringer's solution.

The maximal response given to the first dose might easily give the impression that the sensitisation to duck albumin was as intense as to hen. The record of the second horn, however, shows clearly that complete desensitisation to duck leaves a power of responding even to 0·01 mg. of hen albumin. The same distinction is shown in the next record, in which the non-specific sensitisation is of a high order, but that to the specific antigen is much greater. It may be noted that the bath containing the organ held 50 cc., so that a dose of 0·0001 mg. produces a concentration of only 1 in 500 millions.

Expt. 4. Sensitised with 1 mg. duck. 31st day.

1st horn.	(1) 0·01 mg. Hen. Maximal contraction. Change Ringer.	
	(2) 0·01 mg. Hen. Very small contraction. Change Ringer.	
	(3) 0·01 mg. Hen. Nil.	
	(4) 0·01 mg. Duck. Moderate contraction.	
2nd horn.	(1) 0·0001 mg. Duck. Moderate contraction.	(See Fig. 2.)
	Change Ringer.	
	(2) 0·001 mg. Duck. Nil.	
	(3) 0·01 mg. Duck. Maximal contraction. Change.	
	(4) 0·1 mg. Hen. Nil.	

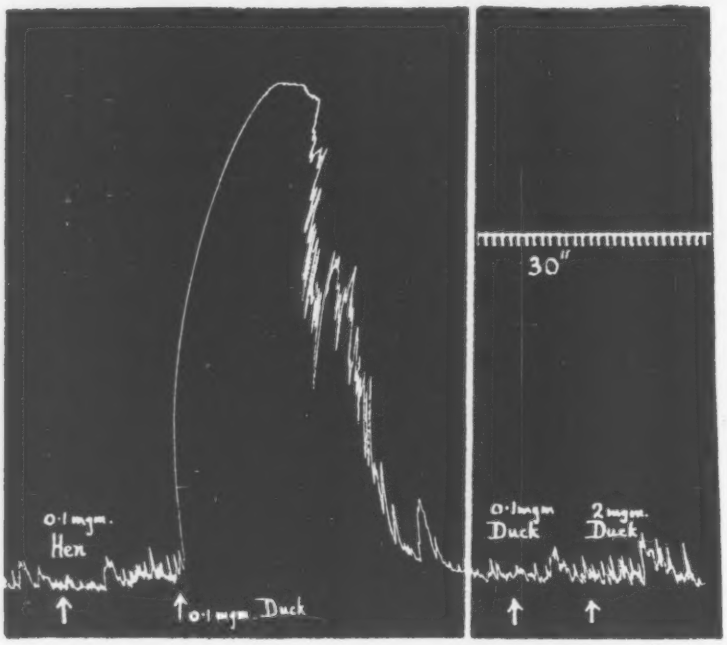

Fig. 3. See Exp. 6, 1st horn. Sensitised with 1 mg. of Duck albumin, 28th day.

Expt. 5. Sensitised with 1 mg. duck. 30th day.

1*st horn.*	(1) 0·001 mg. Hen. Nil.
	(2) 0·001 mg. Duck. Moderate contraction. Change Ringer.
	(3) 0·1 mg. Hen. Nil.
	(4) 0·1 mg. Duck. Larger contraction than at (2). Not maximal.
2*nd horn.*	Not tested.

Expt. 6. Sensitised with 1 mg. duck. 28th day.

1*st horn.* (See Fig. 3.)	(1) 0·1 mg. Hen. Nil.
	(2) 0·1 mg. Duck. Maximal contraction. Change Ringer.
	(3) 0·1 mg. Duck. Nil.
	(4) 2 mg. Duck. Nil.
2*nd horn* (see Fig. 4).	(1) 2 mg. Hen. Moderate contraction. Change Ringer.
	(2) 0·1 mg. Duck. Maximal contraction. Change Ringer.
	(3) 0·1 mg. Duck.
	(4) 2 mg. Duck.

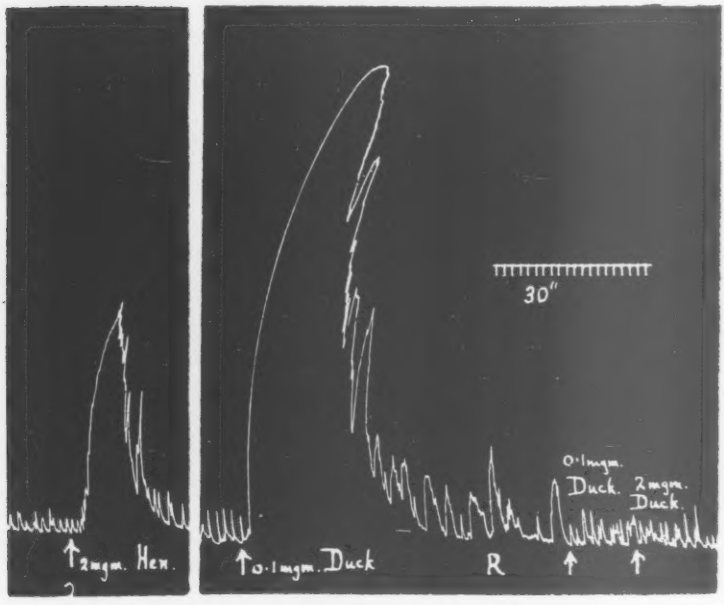

Fig. 4. Continuation of Expt. 6. 2nd horn.

Experiments on Intact Guinea-pigs.

Sensitising injections hypodermic; test injections intravenous.

1. Sensitisation—1 mg. hen albumin.

Weight in g.	Days after first injection	Second dose		Result
300	32	0·1 mg.	Hen	† in 4′
290	30	0·05	Hen	† in 4′
200	30	0·01	Hen	Slight symptoms
180	33	0·01	Hen	Slight symptoms
270	32	0·1	Duck	Moderate symptoms
245	30	0·5	Duck	Slight symptoms

The lethal dose for hen albumin apparently lies between 0·01 and 0·05 mg. For duck albumin it is not reached even at 0·5 mg. though there are symptoms of reaction even with 0·1 mg.

2. Sensitisation—1 mg. duck albumin.

Weight in g.	Days after first injection	Second dose		Result
290	32	0·1 mg.	Duck	† in 3′
250	30	0·05	Duck	† in 3′
225	30	0·01	Duck	Slight symptoms
200	33	0·01	Duck	Slight symptoms
290	32	0·1	Hen	† in 3′
240	30	0·05	Hen	Nil

Again the distinction is clear, but a guinea-pig dies, in this series, after 0·1 mg. of the non-specific albumin.

Discussion.

So far as they go, the results support the conception that the stereochemical structure of the protein molecule is at least an important factor in antigenic specificity. Without the information given by the racemisation method these two proteins would have been indistinguishable, except by an immunological test. The racemisation having shown a structural difference, the presumption that structure and antigenic specificity are related seems to be warranted. This conclusion, however, provides only a first step towards a conception of the relation between antigen and antibody. In due course it will be of interest to discover whether that protein of an immune serum, which carries the specific "antibody" action, shows any difference of molecular pattern from the corresponding protein of a normal serum. Meanwhile the only kind of relation which seems to provide even a distant analogy is that of enzyme and substrate; but the discrimination of the antibody is far more delicately specific.

SUMMARY.

The crystalline albumins from the eggs of the domestic fowl and duck behave as distinct antigens for the anaphylactic reaction. This difference corresponds with a difference in structure, as revealed by the fact that, when the proteins are racemised, the amino-acids escaping racemisation are not identical in the two cases.

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XXVII. THE RÔLE OF THE PLASMA PROTEINS IN DIFFUSION.

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(*Received August 11th, 1919.*)

There is nothing more remarkable than the maintenance of a constant composition of the blood plasma under the most varied conditions. This is shown very clearly in the regulation of the reaction of the blood, and attention has now been directed by Haldane and Priestley [1916] to a similar control as regards the water and salts when large quantities of water or salt solutions are ingested or in the opposite condition of profuse sweating. The electrolyte concentration of the serum was shown by Priestley [1916] to be very slightly diminished when large quantities of water were taken and slightly increased when salts were added to the water. Again, after severe loss of blood, the volume of the circulating fluid is rapidly restored by the entrance of fluid from the tissue spaces and, as will be shown later, this fluid which enters must possess approximately the electrolyte concentration of the original blood plasma. Under these varied conditions there is one class of constituent of the plasma, as there is also one in the tissues, which can only with great difficulty leave its original situation and that is the colloidal element, the protein. Thus after the entrance of non-protein-holding fluids into the blood by ingestion or injection, the colloidal concentration is lowered and on removal of a similar fluid, as in sweating, it is raised. Again after haemorrhage the fluid which enters being either free from or poor in colloid leaves the colloidal concentration at a lower level than the normal. Variations in the colloidal concentration of the blood are therefore dependent upon the addition or withdrawal of non-colloidal solutions. Neither the proteins of the tissue fluids nor the proteins of the plasma can be readily transported, and the exchange of water and salts from one side to the other must be largely governed by the colloidal concentration on these two sides. To Starling and others we owe much of our knowledge of one way in which the protein concentration of a fluid may make itself felt, namely by affecting the osmotic pressure level and so controlling the filtration process through a membrane impermeable for the protein molecule. Before entering upon the consideration of the various ways in which the protein content of a fluid may affect saline diffusion, a brief reference will be made to certain changes which the blood plasma

undergoes after haemorrhage, in so far as these may contribute to our knowledge of the nature of the exchange between tissue fluids and blood. Many investigations have been carried out dealing with this subject and that there are certain well defined changes has been clearly established. After haemorrhage the blood shows a decrease in specific gravity, viscosity, and protein content. The chloride concentration is little if at all affected, while according to Taylor and Lewis [1915] the non-protein nitrogen and urea are apparently increased. Special reference however requires to be made to the degree of the alterations in specific gravity, viscosity, protein and electrolyte concentration with a view to the subsequent consideration of the part played by protein and salts in the exchange between blood and tissues. The effects of haemorrhage therefore were investigated in order to determine the nature and degree of the changes produced in the plasma.

Effects of Haemorrhage.

The methods employed in the determination of these changes were as follows. The specific gravity was estimated in the usual way, a tube of the Sprengel type being employed and the tube filled with water and then with plasma at 18°.

Viscosity was determined by the Ostwald type of viscosimeter and determinations made either at 25° or 37°.

Conductivity was determined by the Kohlrausch method, the conductivity cell used being of the Washburn type and the observations were made in electrically regulated thermostats at 25° and 37°.

Nitrogen was estimated by Kjeldahl's method.

After loss of blood varying from 30—57 per cent. of its total volume, in both the cat and dog, the specific gravity, viscosity and percentage of nitrogen fall while the conductivity rises. The fall in specific gravity is very slight, the mean percentage fall in the cat being 0·2 and in the dog 0·13. The fall in viscosity is more distinct, ranging on an average from a 5 per cent. decrease in the viscosity coefficient in the dog to 10 per cent. in the cat. The nitrogen percentage shows a mean decrease of about 14·5 in the cat to 18 in the dog. The rise in conductivity, although always to be observed, is comparatively small, ranging from 1·92 mean percentage rise in the dog to 3·8 in the cat. These changes are given in detail in the following table (Table I).

The decrease in the nitrogen content of the blood after haemorrhage and the fall in viscosity are the most distinctive alterations, and the latter is the expression of the lowered protein content, as the non-protein nitrogen is evidently increased as a result of haemorrhage. The fluid which enters the blood after haemorrhage must have at least the electrolyte concentration of the normal plasma because even after the most severe loss of blood there is no evidence of a fall in conductivity. It is therefore evident that the blood plasma tends to maintain its normal electrolyte concentration not only after

Table I. *Effects of haemorrhage on blood plasma.*

	Sp. gr.	% fall	Viscosity		$K \times 10^4$ at 37°	% rise	Nitrogen %	% fall
Cat (2·23 kgms.)								
Before haemorrhage	1·0275	—	1·751 (η 25°)	—	127	—	—	—
40 minutes after 60 cc. loss	1·0249	0·25	1·545 „	11·7	131	3·1	—	—
Approximate 30 % loss								
Cat (2·6 kgms.)								
Before haemorrhage	1·0300	—	1·840 „	—	145	—	1·064	—
60 minutes after 120 cc. loss	1·0281	0·18	1·668 „	9·3	—	—	0·927	12·8
90 „ „ „	1·0272	0·27	1·529 „	16·9	154	6·2	0·915	14·0
Approximate 57 % loss								
Cat (2·15 kgms.)								
Before haemorrhage	1·0265	—	1·604 (η 37°)	—	139	—	1·033	—
75 minutes after 50 cc. loss	1·0251	0·13	1·521 „	5·1	142	2·1	0·858	16·9
Approximate 43 % loss								
Dog (18 kgms.)								
Before haemorrhage	1·0249	—	1·593 (η 25°)	—	156	—	0·935	—
3 hours after 500 cc. loss	1·0236	0·12	1·467 „	7·9	160	2·5	0·806	13·7
Approximate 34 % loss								
Dog (13 kgms.)								
Before haemorrhage	1·0250	—	1·409 (η 37°)	—	150	—	0·860	—
40 minutes after 550 cc. loss	1·0237	0·12	1·359 „	3·5	154	2·6	0·691	19·6
2 hours after 550 cc. loss	1·0232	0·17	1·342 „	4·7	151	·66	0·662	23·0
Approximate 52 % loss								

the ingestion of fluids but also after severe loss of blood from the body. As to the nature of the salts entering the blood vascular system, from a small number of analyses it was evident that at least the chloride content of the plasma was not distinctly altered. From Luckhardt's [1910] observations the chloride content of the lymph and tissue fluids is rather higher than that of the blood serum in the same animal. The passage of such a fluid from tissue spaces into blood must affect the protein-salt equilibrium between tissue elements and the fluid which bathes them. It was therefore thought advisable to study by as simple methods as possible the influence of proteins on salt diffusion.

Diffusion of NaCl from Protein Solutions.

In order to study the rôle of proteins in saline diffusion attention was particularly directed to the velocity of diffusion under as nearly as possible constant conditions. The influence of proteins on the diffusion of salt from a protein salt solution into water was investigated in one or other of the following ways. The solutions were separated from one another either by means of a collodion membrane in tubular form, or as a flat diaphragm. The rate of change was followed by making conductivity determinations and also by analysing the saline content of the water at regular intervals. The water towards which the salt was diffusing was either kept running at a constant slow rate or it was changed at stated intervals in order that the concentration gradient should be fixed by the saline concentration in the protein salt solution and therefore be a function of the same. In order that the experimental conditions should be tested as regards their suitability for determining velocity coefficients, the rate of diffusion from a simple aqueous solution of the salt in the same concentration and under exactly the same conditions, as regards filtering membrane, rate of change of water, temperature, etc., was always determined either before or after, or both before and after, the corresponding experiment with the protein salt solution.

All experiments were carried out in one or other of two thermostats heated by electric radiators and with suitable toluene-mercury regulators.

One thermostat was kept at a constant temperature of 25°, the other at 37°, or in some cases slightly over that temperature.

The collodion membranes were made by a modification of Walpole's method [1915], the films being allowed to dry in a closed chamber saturated at 20° with alcohol-ether vapour instead of being freely exposed to air. By this method the passage of the protein through the membrane was diminished to a mere trace.

Conductivity determinations were made by the ordinary Kohlrausch method, the platinised plates being fixed in a definite position in the collodion tube. In the case of the diaphragm dialyser, two glass vessels of the small desiccator cover form (300 cc. capacity) with flanges were used, the collodion

diaphragm with a diffusing surface of 34 sq. cm. being clamped between the two vessels and the edges of the flanges carefully sealed with Chatterton's cement. Tests were always carried out before each experiment in order to exclude any possibility of leakage at the margin. The glass stopper bearing the platinised plates could be placed in either of the two chambers. The majority of the conductivity determinations were made in the fluid from which the salt was diffusing out. The chloride estimations were made by Volhard's method, and in cases where protein was present the modification of this method described by Rappleye [1918] was employed. In the first set of experiments, the diffusion rates of salt into conductivity water from (*a*) simple NaCl solution in water, (*b*) gum arabic solution with the same salt content, and (*c*) ox blood serum were investigated. The experiment was carried out in the diaphragm dialyser, the water being changed at the end of each three hour period and the chloride estimated. The conditions of the experiment in all three cases were identical. In each case the solutions were warmed to 37·5° before placing them in their respective chambers, and the conductivity water introduced at the end of each three hour period was also raised to the same temperature before replacing the preceding dialysate. The fall in conductivity in the three saline solutions was determined at frequent intervals and the one hour readings are given in Table II and are plotted out in Fig. 1.

Expt. 1 (Fig. 1). Diffusion from NaCl, NaCl-gum, and serum to water.

Sol. A. 0·795 per cent. NaCl.
„ B. 0·795 per cent. NaCl in 6 per cent. gum arabic.
„ C. Ox blood serum (0·624 per cent. NaCl).
Temperature 37·5°.

The dialysate was replaced by fresh conductivity water at the end of the third, sixth and ninth hours.

In Fig. 2 the results of the chemical analyses of the chloride content in terms of the percentage NaCl loss during diffusion are plotted; the experiments in each case were for a period of 12 hours.

The viscosity coefficients at 37·5° of the three solutions were

	$\eta_{37·5°}$ (Water = 1.)
A	1·014
B before diffusion	2·676
after „	3·080
C	1·878

As may be readily observed on examination of the curves, the rate of passage out of the salt from the protein solution is evidently not a function of the viscosity, as the gum arabic solution is the more viscous fluid.

From about the fifth—sixth hour the velocity of diffusion from the serum becomes distinctly slower than in the case of the gum arabic or the simple salt solution. The diffusion from the gum arabic solution is somewhat slower and also more regular than in the case of the simple salt solution. If one regards the concentration gradient as determined by the salt concentration

in the solution from which the salt is diffusing and that therefore there is little back effect, then according to the law of mass action the rate of diffusion at the time t should be proportional to the concentration at that time. Taking x_0 as the original concentration in each case and x as the concentration at the stated periods, the velocity coefficients for the time unit one hour, determined from the equation

$$k = \frac{1}{t} \log_e \frac{x_0}{x},$$

are shown in Table II.

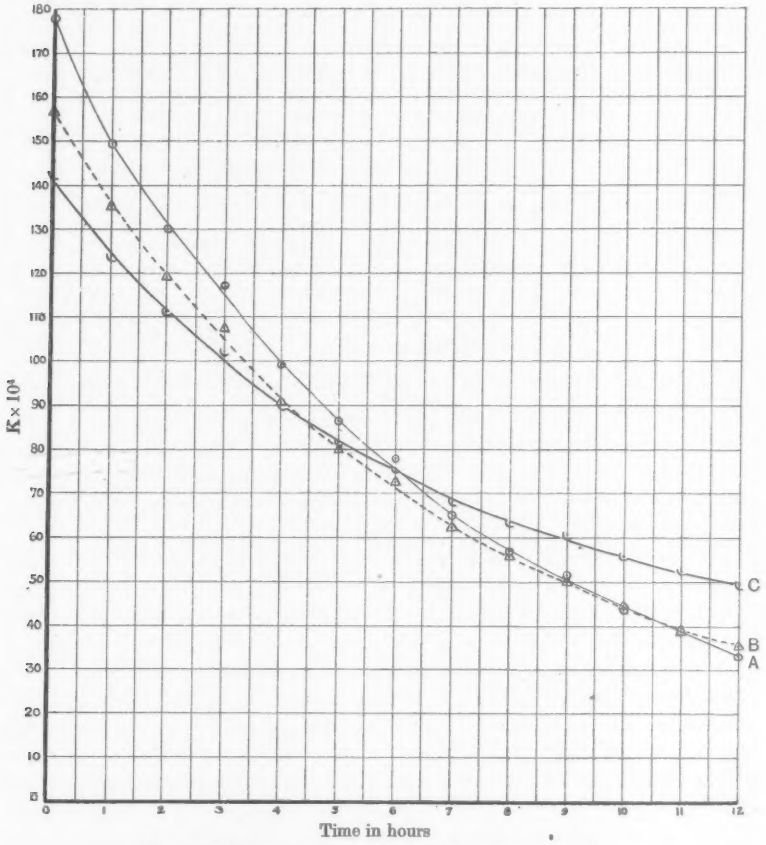

Fig. 1. Diffusion Curves. (Conductivity Measurements).

0·795 % NaCl against Water. ⊙ ——————— A.
Gum Arabic solution in ·795 % NaCl against Water. △ - - - - B.
Blood Serum against Water. ◔ ——————— C.

The coefficients so determined are only approximate, but for comparative purposes between the three solutions they are valid.

Table II. *Velocity coefficients of diffusion in the three solutions.*

A.

Time in hours	NaCl present	$\frac{x_0}{x}$	$k=\frac{1}{t}\log_e\frac{x_0}{x}$
0	1·652 (x_0)	—	—
3	0·986	1·675	0·171
6	0·551	3·000	0·183
9	0·309	5·346	0·186
12	0·131	11·884	0·206

Viscosity $\eta_{37\cdot5^\circ}=1\cdot0146$.

B.

0	1·669	—	—
3	1·018	1·639	0·164
6	0·618	2·700	0·165
9	0·378	4·415	0·166
12	0·228	7·320	0·165

Viscosity $\eta_{37\cdot5^\circ}=2\cdot676$ before and 3·080 after diffusion.

C.

0	1·560	—	—
3	1·037	1·504	0·136
6	0·720	2·166	0·129
9	0·597	2·613	0·106
12	0·481	3·243	0·098

Viscosity $\eta_{37\cdot5^\circ}=1\cdot878$.

In the case of blood serum there is a gradual fall in the coefficient, most distinct between the sixth and ninth hours, the gum arabic saline shows a very regular rate of diffusion proportional to the concentration while the simple watery solution of salt shows a rather variable rate under the experimental conditions due to back pressure effects, but the rate is faster at the lower concentrations.

As the gum arabic solution is the most viscous of the three it is evident as already mentioned that some factor other than that of viscosity is concerned in the diffusion of salt from serum. This of course in no way invalidates the experimental evidence in support of the advantage of the addition of gum to saline injections, the value of which is undoubted as shown by the researches of Bayliss [1918] and others. The blood serum differs from the gum arabic solution in possessing a constituent which requires a certain salt concentration for its solution, namely globulin, and it is during the later period of diffusion that this body separates out. The question whether the other protein in solution in the serum plays any part as a governing factor was examined in the case of dialysed horse plasma. The experiment was carried out in a collodion tube. In order that the effect of the globulin might be compared with the globulin-free (or globulin-poor) solution similar diffusion experiments were carried out with normal and dialysed (10 days) plasma.

The globulin suspension was removed from the dialysed plasma by centrifugalisation. The collodion tube was fitted with a rubber stopper through

which passed two glass tubes carrying platinum plates and also two tubes for filling purposes. The tube was then inserted in a glass jacket, the volume of the outer space being 200 cc. and that of the tube 90 cc. The apparatus was placed in a thermostat at 25°. The solution containing the salt, warmed to 25°, was placed in the inner tube and water, previously heated to the same temperature by passing through spiral glass tubing in the bath, was allowed to

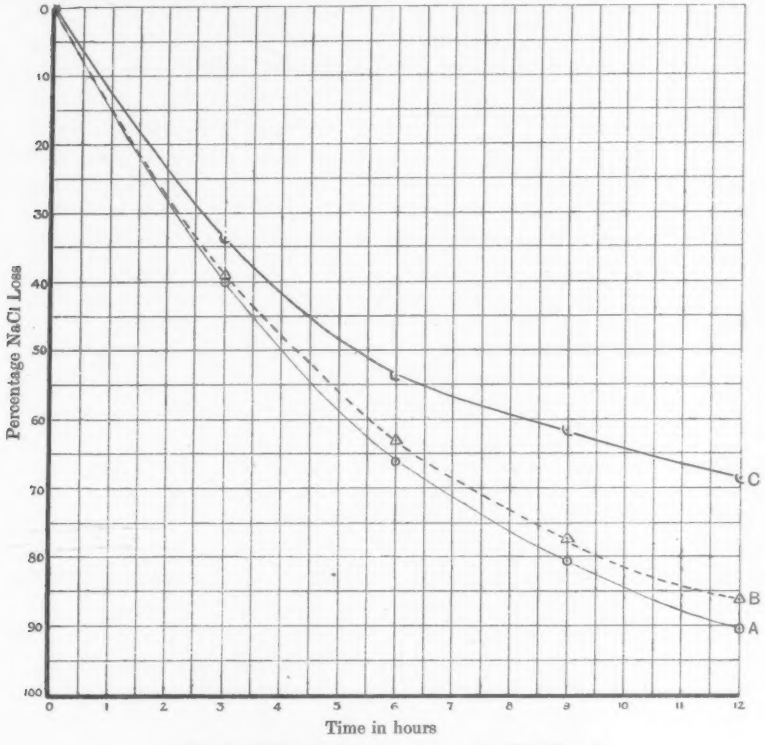

Fig. 2. Diffusion Curves. (Percentage NaCl Loss.)

0·795 % NaCl. ⊙——— A.
Gum Arabic containing 0·795 % NaCl. △ - - - - B.
Blood Serum. (0·624 % NaCl.) ◐ ——— C.

flow through the outer jacket at a rate of 250 cc. per hour. The outflow fluid was collected at two hour intervals and the NaCl estimated. The diffusion rates from the following three salt-containing solutions were then determined and are plotted in Fig. 3 as percentage NaCl loss against time.

Expt. 2. Diffusion from NaCl, normal horse plasma, and dialysed horse plasma (with salt).

Sol. A. 0·547 per cent. NaCl in water.
„ B. Dialysed Plasma containing 0·574 per cent. NaCl.
„ C. Normal Plasma 0·547 per cent. NaCl.

During the first six hours there is not much difference in the rates of diffusion of NaCl from normal plasma and from the simple watery solution of the salt, although the former shows the slower rate. Diffusion from the dialysed horse plasma is distinctly the slowest of the three. Thus 82·96 per cent. is lost from A, 75·57 per cent. from B, and 79·05 per cent. from C.

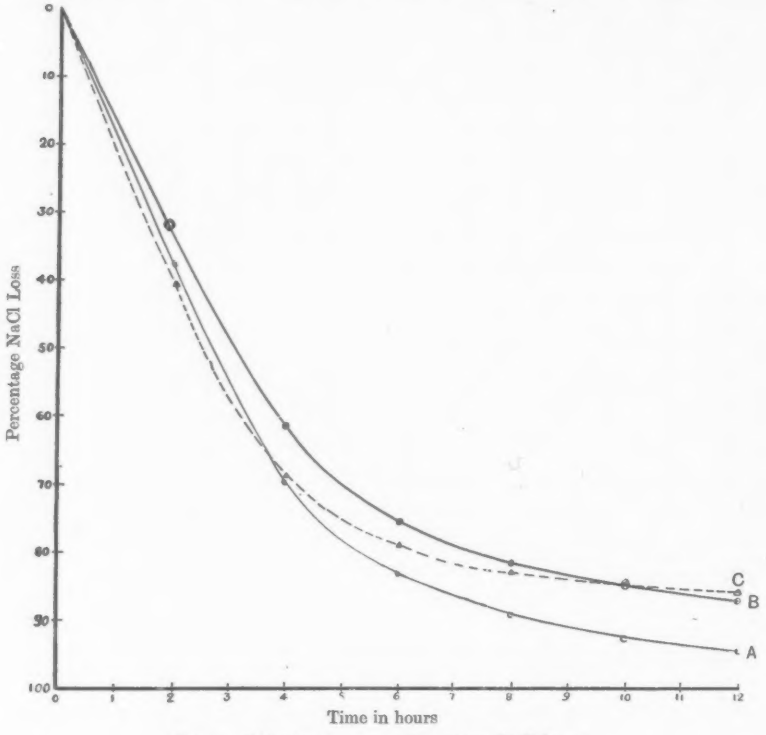

Fig. 3. Diffusion Curves. (Percentage NaCl Loss.)
NaCl Solution ◔ ———— % NaCl = 0·547; A
Dialysed Plasma ⊙ ——— ,, = 0·574; B.
Normal Horse Plasma △ - - - - - ,, = 0·547; C.

In the last six hours however there is a distinct difference between the globulin-free (or poor) solution and the globulin-containing one, the rate of diffusion being slower in the latter than in the former.

If one compares the velocity coefficients of the first four hours and the last eight hours in each of the three solutions, this becomes evident.

	A	B	C
k 1st 4 hours : k last 8 hours	1 : 1·08	1 : 0·92	1 : 0·78

Fig. 3 shows clearly that the curve of diffusion in the case of normal horse plasma begins to show the slowing in the diffusion rate about the fourth hour,

and as diffusion from the dialysed plasma is distinctly slower than from the normal in the preceding four hours the two curves converge from that point onwards.

It is evident therefore as in Experiment 1 that the globulin-holding solution disturbs the diffusion rate in the later stages and as the globulin-free solution in Experiment 2 does not show the same change, the protein other than globulin is not the main cause of the altered rate of diffusion observed in the later stage. As in the case of the blood serum or plasma the fall in diffusion rate is always accompanied by a separation of the globulin, it was thought advisable to investigate the salt diffusion under conditions where the globulin did not separate out on dialysis. The diffusion rates of salt from dialysed plasma and from globulin in acid solution (prepared from the globulin separated out from the normal plasma) were therefore investigated. During the period of diffusion the acid globulin only showed a faint trace of separation from the mixture, the solution becoming faintly opalescent. The globulin obtained by dialysis for a period of ten days from 200 cc. horse blood plasma, was removed by centrifugalisation, dispersed in 5 cc. 0·1*N* HCl, and the volume made up to 200 cc. with water. The mixture was allowed to stand for 24 hours at room temperature and the small residue of undissolved globulin was then removed. The solution was made up to 0·470 per cent. NaCl. The nitrogen value of the solution was 0·146 per cent. The rates of diffusion to water from 0·1 normal NaCl in water, 0·1 normal NaCl in dialysed plasma (nitrogen 0·796 per cent.), and 0·470 per cent. NaCl in the acid solution of globulin were determined. To compare with the acid solution of globulin an alkaline solution was prepared in the same way, 6 cc. 0·1*N* NaOH being added. The nitrogen value of this solution was 0·148 per cent. As the alkali globulin solution on dialysis showed a precipitate it could not be made use of for the purpose of this special experiment, in which the influence of globulin in solution throughout the diffusion process was being investigated. The salt-containing solutions were placed in the collodion tube of the same apparatus, water being allowed to pass through at a rate of 250 cc. per hour, and in addition at two hour intervals the outer fluid was removed and replaced by fresh conductivity water. The water which had run through during the two hour period was added to that drawn off at the end of the period and the chloride content estimated. The results are shown in Fig. 4.

Expt. 3. Diffusion from NaCl, dialysed horse plasma (0·1 normal NaCl), acid globulin (0·470 per cent. NaCl), alkali globulin (0·528 per cent. NaCl) to water.

Sol. A. NaCl, 0·1 normal.
„ B. Dialysed Horse Plasma.
„ C. Acid Globulin.
„ D. Alkali Globulin.

As is evident from the curves in Fig. 4, the diffusion rate from acid globulin is much slower throughout than from the other three solutions which show

diffusion curves of much the same form. The difference in the diffusion rate of salt from the acid globulin solution is possibly related to the influence of the acid on the hydrolysis of the globulin salt, retarding it to such an extent that the solution at the end was only faintly opalescent. Under those circumstances the globulin-salt compound had not evidently undergone dissociation to the same extent as in the alkaline medium. Hardy [1905], Pauli [1903],

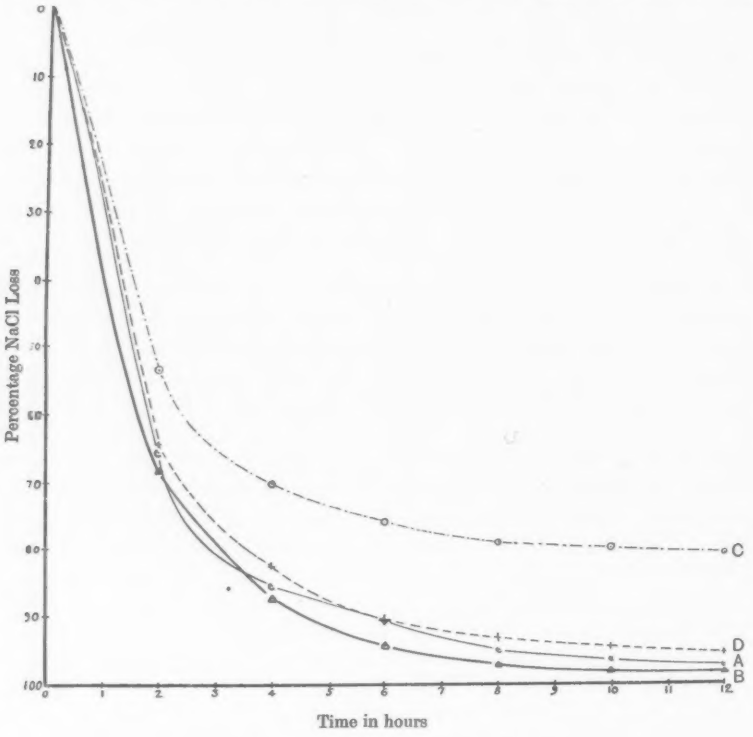

Fig. 4. Diffusion Curves. (Percentage NaCl Loss.)

NaCl Solution ——————— ⊙ % NaCl = 0·585; A.
Serum Globulin (acid solution) –·–·–· ⊙ ,, = 0·470; C.
,, ,, (alkaline solution) – – – – – + ,, = 0·528; D.
Dialysed Plasma ——————— △ ,, = 0·585; B.

Mellanby [1905] and others have studied the influence of salts on the solution or precipitation of the globulin and have recognised that the globulin forms salt compounds in equilibrium with the salt in solution. Pauli concludes that in the solution compounds of globulin with such a salt as NaCl are formed of the type Na-Globulin-Cl. It has been pointed out that, in cases where the effect of electrolytes in solution on living tissues is altered by the addition

of protein to the solution, the action is probably due to the removal of electrolytes from their sphere of action. All such experiments tend to show that certain proteins at least have a chemical (or physico-chemical) affinity for electrolytes.

In order to investigate the matter further certain experiments were carried out to determine the rate of passage of NaCl from an aqueous solution to one of egg white dialysed to such an extent that the globulin had just separated out. The rates at which the salt entered the solution containing a suspension of globulin were determined at first by following the conductivity changes in the protein mixture and also by estimating by chemical analyses the amount of the salt transported to such a solution compared to the rate of transport to water. As the conductivity changes were exactly of the same order as the chemical but were complicated owing to the suspension in the fluid, reference will only be made to the amounts of salt passing from the same volume of 0·03 normal NaCl in one case to the protein mixture and in the other to conductivity water.

Expt. 4. The experiment was carried out in the following way. Filtered egg white was taken, dialysed to the point of separation of the globulin, and 190 cc. of this mixture containing 2·731 per cent. protein were placed, after warming to 37°, in the upper chamber of the diaphragm dialyser. The lower chamber was filled with 300 cc. 0·03 normal NaCl. After a period of four hours had elapsed the fall in NaCl content in the lower fluid was determined.

The total chloride in the lower fluid at the outset was 0·5512 g. in 310 cc. and at the end of four hours, it had fallen to 0·4894, *i.e.* 61·8 mg. NaCl passed to the protein mixture in four hours or 15·45 mg. per hour. Using the same membrane and under the same conditions, but placing conductivity water in the upper chamber, the amount of chloride passing out towards water was found in the experiment to amount to 43·4 mg. in four hours or 10·85 mg. per hour.

The experiment was repeated with a membrane of slightly different permeability, all the other conditions remaining the same, when it was found that 76·27 mg. NaCl passed out to protein in four hours or 19·0 mg. per hour, while to water 52·65 mg. NaCl passed out in four hours or 13·16 mg. per hour. The rate therefore of saline diffusion in the first case to protein and to water respectively was as 15·45 : 10·85 or as 1·42 : 1, and in the second as 19·06 : 13·16 or as 1·44 : 1.

Expt. 5. Using the same membrane that was employed in the last case, the rate of passage of NaCl to dialysed egg white containing 6·08 per cent. protein was tested under exactly the same conditions. It was found that 107·6 mg. NaCl passed out in four hours or 26·9 mg. per hour. The rate therefore in this case with the higher protein content compared to the rate of passage into water was as 26·9 : 13·16 or 2·04 : 1.

Expt. 6. In the next experiment using a fresh diaphragm, the rate of passage of NaCl from a solution of very low cencentration, 0·003 normal, to

an egg-globulin suspension also of low concentration (0·14 per cent. protein) was determined. In this case only 10 mg. NaCl passed through in four hours and in the comparable experiment with the same membrane but with conductivity water in the upper chamber 7 mg. NaCl passed through in the same period, the ratio of the rates therefore being 1·42 to 1.

In all the experiments sodium chloride passed more rapidly from the salt solution to the protein mixture (containing a globulin suspension) than to water. It is evident therefore that the decrease in the diffusion rate of sodium chloride from a salt protein solution such as blood plasma during the period of separation of the globulin is intimately related to the increased rate of passage of salt towards a globulin suspension which has as its accompaniment the solution of the globulin. The question as to whether "mechanical" or chemical affinity is the predominating factor in the fixation of NaCl by globulin (in suspension) is difficult to decide.

Expt. 7. With a view to determine whether the solution of the suspended globulin is the governing factor in the process, the proteins in a specimen of dialysed egg white were coagulated by raising the temperature to 80°. The mixture was then cooled to 37° and 230 cc. of the mixture, containing approximately 4·25 per cent. protein, were placed in the upper chamber of the diaphragm dialyser. The lower chamber was filled with 320 cc. 0·03 normal NaCl. The permeability of the collodion diaphragm was such that with the same strength of the salt solution in the lower chamber and conductivity water in the upper, the respective volumes being the same as in the experiment with the heat-coagulated protein mixture, the rate of passage of NaCl to water was 63·6 mg. in four hours or 15·9 mg. per hour.

In the case of the clotted egg white 64·4 mg. NaCl passed from the salt solution to the protein mixture in four hours or 16·1 mg. per hour. The diffusion rates therefore of salt from simple watery solution to water and to clotted egg white were approximately the same.

It appears therefore that the actual solution of the globulin is the determining factor in governing the rate. That the difference in velocity of diffusion is due to variations in the permeability of the membrane owing to blocking up of the pores with consequent diminution of the cross-sectional area of the diffusion path is unlikely, as in all cases when the globulin can undergo solution salt passes more rapidly to the mixture containing the globulin suspension than to water, and in the case of globulin in acid solution the diffusion of NaCl is delayed throughout the whole period although there is only a slight separation of the hydrolysed globulin. The quantitative relationships between the amounts of protein dissolved and the NaCl fixed, as also the influence of variations in reaction on the process, require further study. It is important however to bear in mind that a protein such as globulin forming a large percentage of the colloidal content of blood plasma, tissue fluids, and tissues possesses a certain salt-retaining power which must be of significance in the maintenance of the chloride content of the body.

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XXVIII. THE EFFECT OF METHODS OF EXTRACTION ON THE COMPOSITION OF EXPRESSED APPLE JUICE, AND A DETERMINATION OF THE SAMPLING ERROR OF SUCH JUICES.

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A number of attempts have been made to follow the changes in chemical composition which fruits undergo during ripening and storage. The number of papers dealing with this subject is large, and many of them are published in obscure journals which are difficult of access. The investigations referred to below may however be regarded as typical in respect of methods, and an examination of these methods serves to show the necessity for the adoption of a standard procedure in the preparation of material for analysis, and for a careful estimate of the limits within which the results obtained are significant.

Most of the earlier work has been directed to following the changes of the fruit in respect of its content of sugar and of acid. In some cases acids and sugars have been estimated in a suspension of the pulp [Bigelow, Gore and Howard, 1905]. In others water or alcohol extracts have been made [Kulisch 1892, Browne 1901, Pfeifer 1876, 1877, 1879]. In the majority of cases, however, the juice has been directly expressed, either before or after killing the tissues [Dixon and Atkins, 1913[1]]. It is clear that the results obtained by methods so various as these cannot be directly comparable. The value of extraction by water or alcohol evidently depends upon the completeness with which it is carried out, and in most of the extractions which have been made by this method, no attempt appears to have been made to determine this point. Davis and Daish [1914] found that it was necessary to extract leaves in a large Soxhlet apparatus for 18—24 hours with boiling alcohol before all the sugars were removed. No corresponding examination appears to have been made in the case of water extracts, but the extraction by cold water of pulp which has been merely cut up or grated must evidently be slow.

There can be no doubt that for most purposes the most satisfactory method of procedure is to press out the sap from pulp which has previously been

[1] See this paper for further references.

frozen. The advantages of this method have been demonstrated by Dixon and Atkins [1913], and though some doubt exists as to certain of their conclusions [Haynes 1919], yet they show clearly that the sap from untreated tissues is more dilute than that from tissues which have previously been exposed to low temperatures, indicating that some or all of the constituents of the sap are held back in some measure by the untreated pulp. It is possible however that tissues which have been frozen, although considerably more permeable than untreated tissues, still hold back some constituents of the juice. Dixon and Atkins made no attempt to ascertain whether this is the case.

The following experiments have therefore been undertaken with the object of determining how far the sap obtained from apples after freezing and pressing is uniform, *i.e.* whether the first runnings from the press are similar in composition to those obtained later, or whether the tissues still hold back certain of the constituents of the sap. Further, in most of the work hitherto published, the amount of divergence due to sampling error is entirely left out of account; indeed in some cases conclusions have been drawn from the analysis of single fruits. Experiments have therefore been made to ascertain the order of the error due to sampling in investigations of this kind.

A third series of experiments was undertaken with the object of ascertaining with what completeness chemical change in the sap was retarded by cold. It was found more convenient to use an ordinary freezing mixture than to freeze with liquid air, but in the first case the process was necessarily slower, and the apple pulp was usually left in the cold for at least 16 hours.

The following chemical and physical properties were investigated, and some or all were used as a basis of comparison in each case.

(1) Value of P_H.

(2) Value of Δ.

(3) Time of fall in viscometer.

(These were all carried out by ordinary standard methods.)

(4) Electrical conductivity (using a Wheatstone Bridge and a Fitzpatrick commutator).

(5) Acidity (titration), using an iodometric method to avoid difficulties arising from the colour of the juice.

(6) Sugars (reducing sugars and cane sugar by Bertrand's method [1906]; the cane sugar was inverted by 10 per cent. citric acid, as recommended by Davis, Daish and Sawyer [1913]).

Methods of Extraction.

The method adopted for extraction was to press out the juice after the tissues had been killed by freezing in an ordinary freezing mixture of ice and salt. A small handpress, worked on the lemon squeezer principle, and provided with a screw, was used. A thin filter cloth was found to be necessary,

and this was saturated with the juice before collection of the sample. The apples were peeled, cut up, and frozen in stoppered jars, and were usually left in the cold for about 16 hours. To ensure uniformity, the frozen pulp was thoroughly mixed before pressing. It was found that the sap obtained by this method from similar samples of pulp gave closely concordant results.

The following points were investigated:

I. *Whether rapid freezing by the use of liquid air produced any alteration in the character of the sample.* It is possible, as has already been stated, that chemical changes may occur during the somewhat prolonged process of freezing in an ordinary freezing mixture. It is also possible that further disruption of the cell membrane may take place when liquid air is used, and that this may affect the character of the sap. For the purpose of comparison, a uniform sample of pulp was divided into two portions, one of which was immediately frozen in liquid air, and pressed out as soon as it had warmed up to the temperature of the laboratory. The other portion was left overnight in a freezing mixture, and then treated in the same way. The chemical and physical properties of the two samples of sap thus obtained were compared.

Table I. *Comparative tests on freezing with liquid air and with a freezing mixture of ice and salt.*

	Liquid air	Freezing mixture
Freezing point depression (Δ)	1·260° C.	1·253° C.
Titration. 10 cc. juice = $N/10$ $Na_2S_2O_3$	9·15, 9·25 } 9·20 cc.	9·25, 9·30 } 9·27 cc.
Conductivity. $c \times 10^5$	245	229
Time of fall in viscometer	41·2″	41·2″
Total sugar (after inversion)	8·63 %	8·57 %

It will be seen that the differences obtained by using liquid air instead of a freezing mixture are very small. It is only in the case of electrical conductivity that the difference rises above the limits of experimental error. The reason for this difference has not at present been investigated.

II. *Whether the tissues after freezing are freely permeable to all those constituents of the cell sap present in the expressed juice.* For this purpose the juice was pressed out in several fractions, and these were examined separately. The results are shown in Table II.

In this case again the greater number of the results obtained were uniform. The concentration of acids and of sugars was found to be the same in the first fraction of juice from the press as in the last. The viscosity however varied greatly, indicating that the colloidal constituents of the sap are held back by the tissues. A corresponding difference was also observed in the amount of the precipitate obtained from the two fractions by the addition of alcohol. This precipitate is mainly pectin, which is the principal colloidal constituent of apple-juice.

Table II. *Results of fractional pressing.*

	Fraction	P_H	Δ °C.	Time of fall (viscometer)	Reducing sugars %	Total sugars %
	1	3·83	1·44	5′ 24″	—	—
Series I	2	3·84	1·42	0′ 55″	—	—
(unripe)	1	3·84	1·43	3′ 34″	10·10	11·40
	2	3·85	1·42	1′ 35″	9·97	11·35
Series II	1	3·87	1·60	3′ 43″	10·9	11·75
(ripe)	2	3·82	1·60	1′ 59″	11·05	11·65

	Fraction	Time of fall (viscometer)
	1	4′ 17″
	2	0′ 54″
Series III (unripe)	1	5′ 29″
	2	2′ 59″
	3	1′ 20″

	Fraction	Δ °C.	Titration	Conductivity ($c \times 10^5$)	Reducing sugars	Total sugars
Series IV	1	1·110	11·3	241	6·20	6·99
(cold store)	3	1·112	11·15	238	6·25	7·08

	Weight of alcohol-precipitate from 10 cc. juice
1	0·0292 g.
3	0·0090 g.

	Fraction	Δ °C.	Titration	Conductivity ($c \times 10^5$)
Series V	1	1·377	10·7	237
(ripe)	3	1·380	10·7	239

III. *The probable error due to individual variability in the apples used.* This was estimated in the usual way by the comparison of a number of samples of sap obtained from individual apples.

The apples on which these experiments were carried out had been kept in cold store for some months, and it is possible that they varied among themselves more than would have been the case had they been more recently picked. Several sets of experiments were made for this purpose. As the results obtained were very similar, it has been thought sufficient to give one set of results, the probable errors being calculated from ten observations[1].

These have been calculated for the purpose of attaining a definite standard of accuracy defined as the probability that the error of any observation may lie within certain limits. This is determined by the number of samples taken.

The standard probability will be taken here as 0·957 (22 to 1), *i.e.* three times the probable error, and the limit below which differences cease to be significant as 5 per cent.

[1] A non-mathematical treatment of the use of probable errors applied to biological work is given by Wood [1911].

Table III. *Probable error attaching to various measurements.*

No. of sample	Freezing point depression (Δ) °C.	Titration	Conductivity ($c \times 10^5$)
1	1·265	9·85	223
2	1·173	9·65	223
3	1·143	12·7	287
4	1·245	10·05	233
5	1·150	10·55	250
6	1·290	8·3	240
7	1·323	10·3	217
8	1·243	10·45	234
9	1·225	10·95	252
10	1·238	9·2	208
	Mean = 1·230 ± 0·0127	Mean = 10·20 ± 0·25	Mean = 236·4 ± 4·8

The probable errors of a single observation are:

		%
Δ	0·0412	= 3·2
Titration	0·78	= 7·7
Conductivity	15·2	= 6·4

Since the probable error of the difference of the observations is equal to $\sqrt{2}p$, where p is the probable error of either observation, the above conditions are satisfied by the equation

$$\frac{3 \times \sqrt{2}p}{\sqrt{n}} = 5,$$

where n is the number of observations. The number of samples which must be taken in order that there may be a probability of 0·957 that a 5 per cent. difference is significant is therefore given by the equation

$$n = 2\left(\frac{3p}{5}\right)^2.$$

The following are the values of n obtained from this equation:

For Δ	7·4
For titration	42·6
For conductivity measurements	29·4

The least number of samples required for these three estimations is therefore 8, 43, and 30 respectively.

These are large numbers and it is to be remembered that the standard of probability assumed is somewhat low. It is more usual to require a probability of not less than 0·969 (30 to 1), *i.e.* a difference of 3·2 times the probable error, in which case the necessary number of samples would be 9, 49, and 34 respectively.

The value of the probable error deduced from such a small number as ten samples is, of course, only approximate but it shows clearly how important is the sampling error. The use of more samples would probably give a somewhat larger probable error but the labour involved in working even with ten samples is considerable.

The importance of the sampling error in measurements of this kind does not appear to have been fully realised by previous workers. It is possible

that the apples used in these determinations differed among themselves more than would have been the case had they been subjected to different treatment, and the error would probably have been considerably reduced if all the apples had been obtained from a single tree, since Kulisch [1892] has shown that apples from different trees may exhibit marked and constant differences; but whether this be so or not it is clear that the sampling error may be very large and from the nature of the material must be far from negligible in any experiments of this kind. Where experiments have been made on two or three fruits only, there can therefore be little doubt that the differences observed are not significant unless they are very large.

Summary.

The juice obtained from apples after freezing has similar properties, whether the tissue is frozen rapidly by means of liquid air, or slowly by a freezing mixture.

The sugars and acids present in the tissues can be readily pressed out from the frozen and thawed pulp; colloidal substances, however, of which pectin is the most important, are held back to a large extent by the pulp, so that the amount of these substances present in the expressed juice is no measure of the quantity in which they are present in the tissues.

The samples investigated varied considerably, especially in acid content. These large fluctuations cause the probable error to be very large, and the number of samples required to give results significant within 5 per cent. for lowering of freezing point, for titration, and for conductivity, was 8, 43, and 30 respectively, when the standard of probability assumed was 0·957 (*i.e.* a probability of 22 to 1). The neglect of sampling errors in previous estimations of this nature detracts very seriously from the value of the results obtained and renders many of them worthless.

The work described above has been undertaken in connection with investigations of Cold Storage problems which have been carried out for the Food Investigation Board of the Department of Scientific and Industrial Research. We are indebted to Professor V. H. Blackman for suggestions on which this work has been largely based, and also for much help and advice.

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XXIX. A COMPARISON BETWEEN THE PRECIPITATION OF ANTITOXIC SERA BY SODIUM SULPHATE AND BY AMMONIUM SULPHATE.

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(*Received August 13th, 1919.*)

My previous work on the factors influencing the concentration of sera has led me to the conclusion that the preliminary heat-denaturation so generally advocated prior to the fractional precipitation of the serum proteins by ammonium sulphate and by sodium chloride does not of necessity materially contribute to the refining and concentrating of antitoxic sera. Recent experiments have shown that, with the technique adopted for the routine work, the heat-denaturation merely induces the precipitation of the antitoxin-bearing protein at lower concentrations of the electrolyte than would be required in the unheated plasma; apparently the heating does not affect the nature of the antitoxin-protein combination.

Further corroborative evidence on this point has been furnished by a study of the fractional precipitation of the serum proteins and of the antitoxin from unheated and from heated antidiphtheritic plasma by sodium sulphate[1].

Several series of experiments were undertaken with samples of oxalated plasma from individual horses and from batches of pooled plasma from different horses. In all cases the plasma was adjusted, either by bringing the $[H^{\cdot}]$ to definite values, or by the addition of cresylic acid in stated amounts to the plasma. The method of procedure for each of the series of experiments was as follows: equal volumes of the adjusted plasma were measured into stoppered bottles each of which contained the amount of anhydrous sodium sulphate necessary to bring the proportion of the latter in solution in the plasma to values ranging from 4 to 34 per cent. Owing to the slight solubility

[1] Some years ago Dr C. J. Martin (unpublished observations), worked out a method for the concentration of antitoxic sera by the fractional precipitation of the serum proteins with sodium sulphate rather than with the more toxic ammonium sulphate. Taking advantage of the slight solubility of sodium sulphate at 0° he was able to freeze out the excess of this salt from the Second Fraction precipitates thereby dispensing with the lengthy process of dialysis. For some reason the end products sometimes showed too high a viscosity. The factors underlying this variability were not fully investigated as, in the meantime, Gibson and Banzhaf published their methods involving the heat-denaturation of the proteins.

of anhydrous sodium sulphate in water[1] the respective liquids were kept at 35—40° and constantly stirred until the solution of the sulphate had been effected. Samples were then taken from the bottles and filtered.

The filtration of the hot liquids containing percentages of sodium sulphate in solution greater than those required for saturation at room temperature should be conducted in funnels surrounded with jackets at a temperature of from 30—35°. However, owing to the tendency of solutions of sodium sulphate to remain supersaturated, the filtration of plasma containing 18 per cent., or less, of the anhydrous sulphate could be conducted at the ordinary spring and summer temperature of our London laboratories (about 17°) without the crystallisation of Na_2SO_4, $7H_2O$ from the mixtures or from the filtrates. It was always advisable to filter large quantities of the plasma containing the higher concentrations of the sulphate through funnels jacketed at 30—35°. The bottles containing the sulphated plasmas were heated in a water bath at 57·5—58° for five to six hours and the hot liquids were filtered, the filtering funnels being jacketed at 35—40° when necessary.

The antitoxin and the protein contents of the filtrates from these heat-denaturated sulphated plasmas were estimated in the usual way, and, after the necessary volume corrections, the data thereby obtained were expressed in the form of curves which were compared with those obtained from similar estimations of the filtrates of the unheated liquids and also with those from the precipitation of samples of the same plasmas by ammonium sulphate.

In accordance with the recent request for the curtailment of the subject matter of communications, curves expressing the experimental data from only three separate batches of antidiphtheritic plasmas are included in this paper.

I. The precipitation of the serum proteins by sodium sulphate and by ammonium sulphate.

Gravimetric estimations of the percentage composition of the respective plasmas were made as regards total protein, pseudoglobulin and albumin with the following results.

	A	B	C
Total protein	7·92 %	8·90 %	7·60 %
Pseudoglobulin	3·18	3·52	2·74
Albumin	2·75	2·68	2·13
Euglobulin, fibrin (by difference)	2·00	2·68	2·73

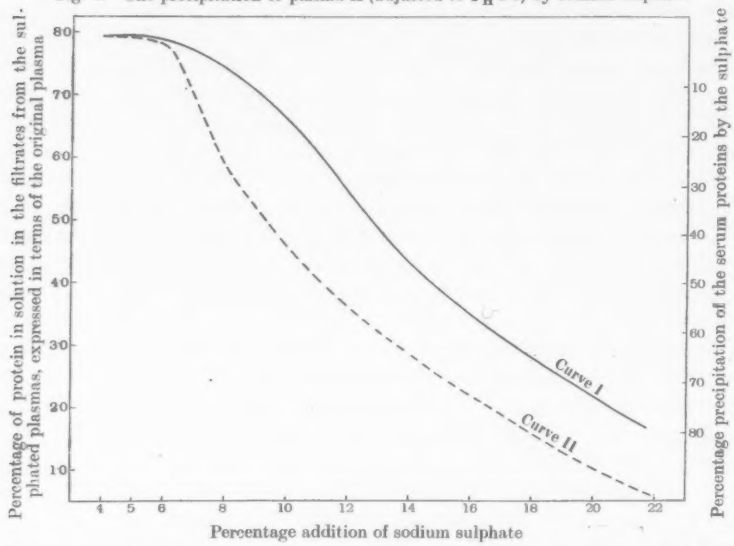
[1] 100 parts of water at $t°$ dissolve a parts of anhydrous Na_2SO_4:

$t°$	a parts Na_2SO_4
0	5
13	11·8
16	14·5
20	19·5
30	43

The variations in the proportionate composition of the proteins of the respective plasmas can doubtless be ascribed to variations in the stage of immunisation or of resting at which the horses were bled.

The data obtained for the sodium sulphate precipitation of the plasma A (P_H 8·3) and of the cresylised plasmas A and B, and for the ammonium sulphate precipitation of the plasma A (P_H 8·8) and of the cresylised plasma B, have been represented in the curves in Figs. 1, 2, and 4, and 3 and 5 respectively. In each figure the continuous and the intermittent curves represent the data for the unheated and the heated sulphated plasmas respectively.

Fig. 1. The precipitation of plasma A (adjusted to P_H 8·3) by sodium sulphate



The continuous Curve I ———— represents the precipitation of the plasma A adjusted to P_H 8·3

The intermittent Curve II — — — represents the precipitation of the plasma A adjusted to P_H 8·3 and subsequently heated to 57·5° C. for 5 hours

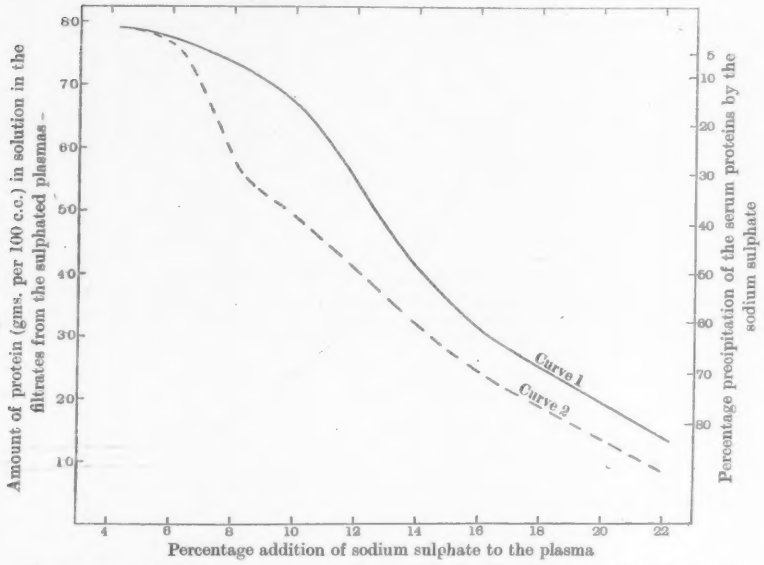
From the relative positions of the points on the curves can be deduced (*a*) the amount of protein remaining in every 100 c.c. of the filtrates from the sulphated plasmas, and (*b*) the percentage precipitation of the serum proteins at each stage with sodium sulphate.

It will be seen that the gradients of the curves for the precipitation of the serum proteins from unheated plasma by sodium sulphate are similar to those for the precipitation of the same plasma by ammonium sulphate; with neither salt are there critical points for the precipitation of eu- or pseudo-globulin or of the serum albumin.

It was found that at all stages in the precipitation of the heated plasmas with sodium sulphate there was a marked increase in the precipitability of

the serum proteins similar to that shown by the precipitation of the same plasmas with ammonium sulphate. Moreover, the increased precipitability of the pseudoglobulin by sodium sulphate, registered by the relative shift in the position of corresponding points on the pairs of curves in Figs. 1, 2, and 4 was regulated by the reaction of the plasma, by the temperature at which the plasma was heated and by the duration of the heating at the chosen temperature.

Fig. 2. Showing the precipitation of the serum proteins from plasma A, containing 0·30 per cent. of cresylic acid, by sodium sulphate


Amount of protein (gms. per 100 c.c.) in solution in the filtrates from the sulphated plasmas
Percentage precipitation of the serum proteins by the sodium sulphate
Curve 1
Curve 2
Percentage addition of sodium sulphate to the plasma

The continuous Curve 1 ———— represents the precipitation of the unheated cresylised plasma A

The intermittent Curve 2 — — — represents the precipitation of the same cresylised plasma after it had been heated to 57·5° C. for 5 hours

On the other hand, in the euglobulin-pseudoglobulin zone the extent of the shift of corresponding points on the curves for the precipitation of the respective heated and unheated plasmas by the two sulphates is greater than that shown in the pseudoglobulin area and than that shown by the data from the precipitation of similarly adjusted solutions of pseudoglobulin [Homer 1919].

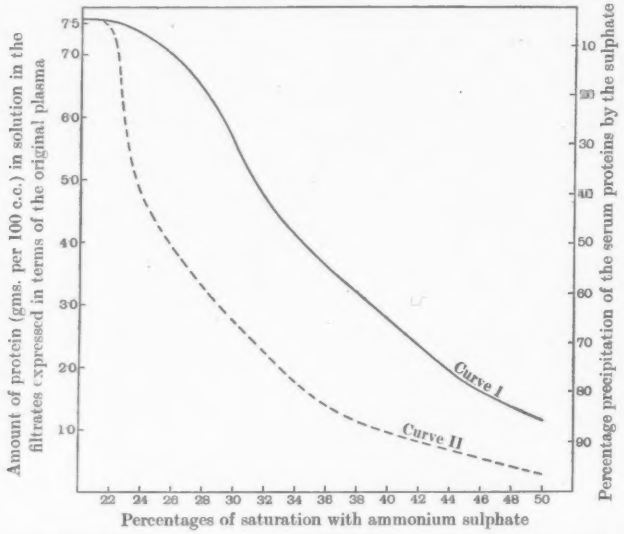
No doubt this differentiation is due to differences in the relative susceptibility of the individual serum proteins to heat-denaturation.

As was to be expected from the general behaviour of colloids the precipitation of the individual proteins from plasma began at and was completed at lower concentrations of ammonium or of sodium sulphate than was

required for their precipitation from their respective separate solutions in saline; there was also a more marked overlapping of the precipitation limits for the individual proteins than was found for their separate solutions.

Thus, from solution in normal saline, the bulk of the pseudoglobulin is precipitated between the limits of 32 and 50 per cent. of saturation with ammonium sulphate, and between 13 and 23 per cent. of anhydrous sodium sulphate; the albumin under similar conditions is mainly precipitated between 54 and 76 per cent. of saturation with ammonium sulphate and between 20 and 34 per cent. of sodium sulphate.

Fig. 3. The precipitation of plasma A (adjusted to P_H 8·8) by ammonium sulphate

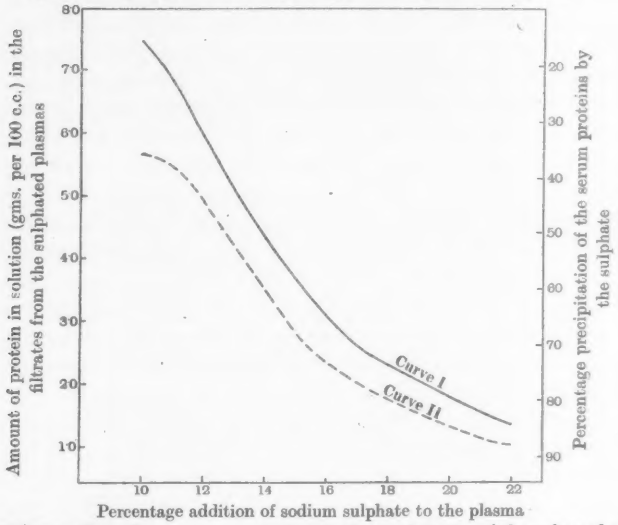


The continuous Curve I ———— represents the precipitation of the unheated plasma A (P_H 8·8)

The intermittent Curve II — — — represents the precipitation of the plasma A (P_H 8·8) after it had been heated to 57·5° C. for 5 hours

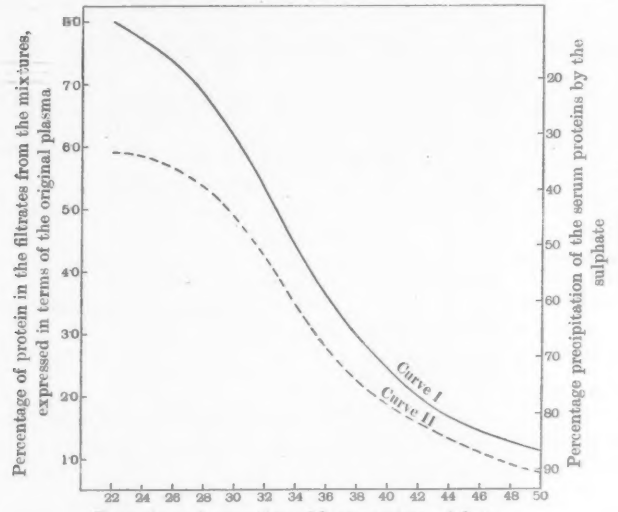
On the other hand there was an appreciable precipitation of pseudoglobulin from unheated unadjusted plasma (P_H 7·4) on the addition of ammonium sulphate to the extent of 28 per cent. of saturation or of 11·5 per cent. of anhydrous sodium sulphate. Moreover, while the precipitation of the pseudoglobulin from the plasma was complete at 46 per cent. of saturation with ammonium sulphate or on the addition of 18·5 per cent. of sodium sulphate, there was, even at these concentrations of the sulphates, an appreciable precipitation of albumin. The precipitation of the latter from the plasma was complete at 67·5 per cent. of saturation with ammonium sulphate or at a concentration of 32 per cent. of anhydrous sodium sulphate.

Fig. 4. The precipitation of cresylised plasma B by sodium sulphate


Amount of protein in solution (gms. per 100 c.c.) in the filtrates from the sulphated plasmas
Percentage precipitation of the serum proteins by the sulphate
Curve I
Curve II
Percentage addition of sodium sulphate to the plasma

The continuous Curve I ———— represents the precipitation of the unheated plasma
The intermittent Curve II — — — represents the precipitation of plasma after it had been heated at 57·5° C. for 5 hours

Fig. 5. The precipitation of cresylised plasma B by ammonium sulphate

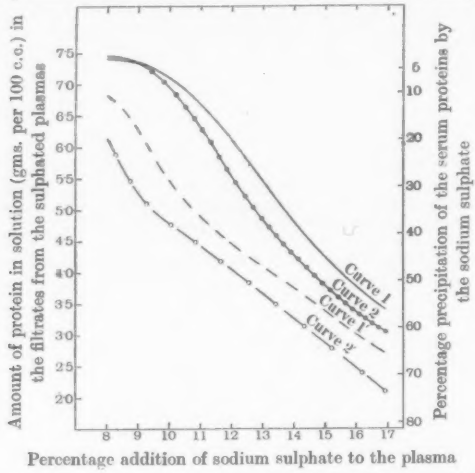

Percentage of protein in the filtrates from the mixtures, expressed in terms of the original plasma
Percentage precipitation of the serum proteins by the sulphate
Curve I
Curve II
Percentage of saturation with ammonium sulphate

The continuous Curve I ———— represents the precipitation of unheated cresylised plasma B
The intermittent Curve II — — — represents the precipitation of cresylised plasma B after being heated at 57·5° C. for 5 hours

For some time it has been realised that the denaturation of the serum proteins considerably affects their precipitability and that the extent of the overlapping of their precipitation limits from heat-denaturated plasma depends largely on their respective susceptibilities to heat-denaturation.

Recent work has shown that, even with unheated plasma, there are several factors affecting the extent to which the various serum proteins are precipitated by the sulphates under discussion. In this connexion reference may be made to the results which have accrued from preliminary experiments as regards the influence of dilution, of reaction and of the addition of substances such as phenol and cresylic acid to plasma prior to its precipitation by electrolytes.

Fig. 6. Showing the influence of cresylic acid on the precipitability of the serum proteins from plasma and from heat-denaturated plasma by sodium sulphate



Percentage addition of sodium sulphate to the plasma

The continuous Curve 1 ———————— represents the precipitation of protein from plasma C (P_H 7·3)

The continuous Curve 2 —o—o—o represents the precipitation of protein from plasma C containing 0·30 per cent. of cresylic acid

The intermittent Curve 1′ — — — represents the precipitation of protein from plasma C (P_H 7·3) heated at 57·5° C. for 5 hours

The intermittent Curve 2′ — o — o — o represents the precipitation of protein from cresylised plasma C heated at 57·5° C. for 5 hours

It has been found that the limits stated above for the precipitation of the serum proteins were consistent for all the samples of plasma examined in which the protein content varied from 6·5—10 per cent. Dilution of the plasma with water or with normal salt solution did not necessitate a change in the concentration of the precipitating electrolyte until the percentage of protein in solution was reduced to the order of 2·5. At this stage a concentration of 70 per cent. of saturation with ammonium sulphate or of 34 per cent.

of sodium sulphate was required before complete precipitation of the serum protein was effected. The further dilution of the plasma required a further increase in the concentration of the precipitating salt.

The required concentration of the precipitating sulphate was also affected by the reaction of the plasma. However, the variations in the precipitability of the proteins when the reaction of the plasma lay between P_H 5·2 and 9·3 were slight; in more acid or more alkaline plasmas greater changes were detected and, as was to be expected, those shown in plasma more acid than P_H 5·2 were more marked than those found in the alkaline plasma.

The addition of cresylic acid (0·30 per cent.) to unheated and to heat-denaturated plasma produced marked changes in the precipitability of the serum proteins at all stages with the given sulphates. This phenomenon is readily seen from a comparison of the curves in Fig. 6. The curves 1 and 1′ respectively represent the precipitation of unheated and of heat-denaturated plasma C by sodium sulphate; the curves 2 and 2′ represent the corresponding treatment of plasma C containing 0·30 per cent. of cresylic acid.

The data included in Table I show that the increased precipitability of the serum proteins in cresylised plasma is a function of the amount of cresylic acid present in the plasma.

Table I. *Showing the effect of the addition of varying amounts of cresylic acid on the precipitability of the serum proteins in unheated antidiphtheritic plasma.*

Percentage addition of cresylic acid to the plasma	Concentration of (A) anhydrous sodium sulphate and (B) ammonium sulphate required for the complete precipitation of the serum proteins from the plasma			
	A Na_2SO_4 expressed as		B $(NH_4)_2SO_4$ expressed as	
	grams per 100 c.c.	gram-molecules per 100 c.c.	Percentage of saturation	gram-molecules per 100 c.c.
0·00	32	0·225	68	0·24
0·15	30	0·21	61·5	0·215
0·30	27	0·19	58·5	0·20
0·45	26	0·18	56	0·19
0·60	25	0·176	54	0·18
0·75	24	0·17	51	0·175

It was also interesting to find that, under the conditions of the experiment, the values for the molecular concentrations of ammonium sulphate required for the complete precipitation of the proteins from the respective plasmas are practically the same as those for sodium sulphate.

The evidence so far obtained in regard to the precipitability of the proteins of unheated and of heat-denaturated plasma indicate that the addition of cresylic acid or of phenol to the extent of 0·5 per cent. or less prior to the prolonged heating of the plasma at 57·5° for five hours does not increase the extent of the heat-denaturation of the serum proteins. The cresylic acid and the phenol materially aid the precipitation of the heat-denaturated protein

which is apt to be so troublesome in the filtration of concentrated antitoxic sera; in this respect their behaviour is analogous to that shown by electrolytes. [Homer 1918, 1.]

In contrast to the experience with non-cresylised plasma, it was found that the dilution of cresylised plasma to a volume four to five times that of its original volume, provided the percentage of cresylic acid in solution were kept constant, did not affect the precipitability of the serum proteins. On the other hand, if, by the dilution, the percentage of cresylic acid in solution were reduced to a value X, then the amount of sodium sulphate or of ammonium sulphate necessary to precipitate the protein was found to be the same as that required by the original undiluted plasma containing X per cent. of cresylic acid.

The above observations on the variations in the precipitability of the proteins of unheated plasma present many points of interest for discussion and elucidation.

Shortly I hope to publish more extensive quantitative data in this connexion.

II. The association of antitoxin with the proteins precipitated by the addition of ammonium sulphate or of sodium sulphate to antidiphtheritic plasma.

Recent work on the precipitation of solutions of pseudoglobulin by ammonium sulphate [Homer 1919], has demonstrated that there is a comparatively small load of antitoxin attached to the proteins precipitated by ammonium sulphate in the pseudoglobulin-euglobulin zone, that the remainder of the antitoxin is evenly distributed throughout the fractions of the pseudoglobulin precipitated at higher concentrations, and moreover that, at all stages in the latter region, the increased precipitation of proteins due to heat-denaturation is a linear measure of the accompanying increased precipitation of antitoxin.

These results together with my previous observations on the factors influencing the concentration of heat-denaturated plasma by ammonium sulphate have led me to the conclusion that the ratio of antitoxin to protein in the final products can only be increased by a removal of the proteins to which little or no antitoxin is attached, viz. those of the euglobulin-pseudoglobulin or of the pseudoglobulin-albumin zones.

Further evidence in this respect is given by the fractional precipitation of the unheated plasma by ammonium sulphate and by sodium sulphate respectively.

In Table II have been included the data obtained from the fractional precipitation of separate volumes of unheated antidiphtheritic plasma A and of the same plasma to which an addition of 0·30 per cent. of cresylic acid had been made. The method of procedure was the same as that used in the fractionation of pseudoglobulin [Homer 1919].

Table II. *The fractional precipitation of unheated anti-diphtheritic plasma by ammonium sulphate*.*

Limits of saturation with ammonium sulphate between which the protein fractions were isolated	Percentage of the total protein appearing in the dialysed fraction	Percentage of the total antitoxin appearing in the dialysed fraction	Degree of concentration found in the end products, calculated, for purposes of comparison, on the basis that they contain 20 % of protein
	Non-cresylised Plasma.		
33–50	45·5	77	4·2
36–50	37·0	70	4·95
38–50	23·3	48	5·45
36–44	22·4	60	7·1
38–44	10·5	38	9·5
	Cresylised Plasma.		
32–45	40·8	70	3·8
33–45	34·1	68	5·1
34–45	25·8	62	5·4
36–45	21·0	51	5·5
38–45	10·4	28	6·0
34–50	27·3	62	5·1

* The antitoxin precipitated with the various First Fraction precipitates was completely recovered by extraction of the precipitates with a saturated solution of salt.

Table III. *The fractional precipitation of unheated cresylised antidiphtheritic plasma by sodium sulphate*.*

Limits of percentage addition of sodium sulphate to the plasma between which the protein fractions were isolated	Percentage of the total protein appearing in the dialysed fraction	Percentage of the total antitoxin appearing in the dialysed fraction	Degree of concentration found in the end products, calculated, for purposes of comparison, on the basis that they contain 20·% of protein
8–16·5	59·5	97	3·6
10–16·5	53·0	97	4·3
11–16·5	47·0	97	4·6
12–16·5	36·0	80	5·0
13–16·5	26·0	60	5·5
14–16	14·6	40	6·5
10–18	57·0	97	3·7
10–20	63·0	97	3·45

* The antitoxin precipitated with the various First Fraction precipitates was recovered by extraction with a saturated solution of sodium chloride.

In Table III have been embodied the corresponding data obtained from the fractional precipitation of unheated cresylised plasma B by sodium sulphate. The method of procedure was as follows.

To separate volumes of cresylised plasma (0·30 per cent.) was added the amount of anhydrous sodium sulphate necessary to bring the concentration of the latter to the value chosen for the precipitation of the First Fraction,

viz. X per cent. The respective liquids were kept at a temperature of 35—40°[1] and were constantly stirred until complete solution of the sulphate had taken place. The hot liquids were then filtered through paper at room temperature. The First Fraction precipitates were washed with a volume of an X per cent. solution of anhydrous sodium sulphate in water equal to that of the plasma taken for fractionation, the washings were filtered and added to the main filtrate. To the measured volume of filtrate and washings was added the weight of anhydrous sodium sulphate necessary to bring the concentration of the latter in solution up to Y per cent.[2] The liquids were again heated to 35—40° and kept at this temperature until complete solution of the sulphate had taken place. The hot liquids were then filtered, and as before (*ante*, p. 279), where the concentration of sulphate was 18 per cent. or less, the filtration was conducted at room temperature; with greater concentrations of the sulphate it was necessary to enclose the filtering funnels in water jackets kept at about 35°.

Where the value for Y was not greater than 18 per cent. the protein fractions, thus isolated in the Second Fraction precipitates, were drained and pressed quickly in the usual way. At greater concentrations of the sulphate, owing to the tendency of the sodium sulphate to crystallise out from the fluid adhering to the precipitate, it was necessary to conduct the pressing operations in the hot room.

The pressed precipitates were dialysed and to the residues from dialysis were added 0·35 per cent. of cresylic acid, and 1 per cent. of sodium chloride. The protein and antitoxin content of the end products were respectively estimated and from the results were calculated the degree of concentration and also the percentages of the total protein and of the total antitoxin associated with the various fractions.

The data included in the tables show that, by a suitable selection of the precipitation limits for the isolation of the antitoxin-protein fraction, results can be obtained from unheated plasma similar to those hitherto obtained from heated plasma, provided that the precipitating limits be chosen so as to exclude albumin, euglobulin and the lower fractions of pseudoglobulin to which a relatively small proportion of the antitoxin is attached.

More detailed work was then undertaken to furnish evidence both as to the proportional distribution of the antitoxin throughout the protein fractions precipitated at progressively increasing concentrations of sodium sulphate, and also as to whether the heat-denaturation (at 58°) effects changes in the nature of this association.

Several batches of plasma were treated in the manner described in Section I (p. 279). Estimations of the protein and the antitoxin content of the filtrates

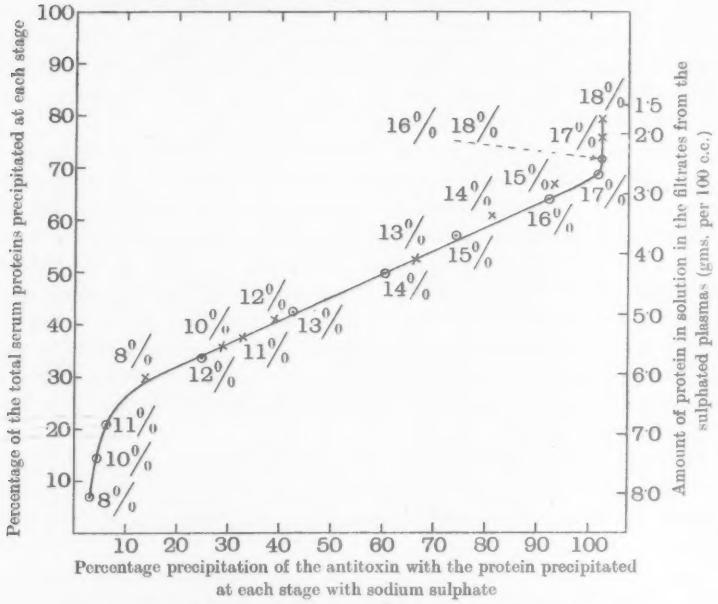
[1] No appreciable heat-denaturation of the serum proteins takes place during the short time required for the solution of the sulphate.

[2] To every 100 c.c. of liquid add $Y - X$ grams anhydrous sodium sulphate; the volume changes following the solution of the sulphate in the plasma can be neglected.

from the unheated and from the heated sulphated plasmas were made and from the data thus obtained were calculated (*a*) the amount of protein remaining in every cc. of the filtrates, and (*b*) the respective precipitations of the serum proteins and of the antitoxin at each stage with the sulphate.

It was found that in the *unheated* plasmas the preliminary treatment of the plasma affected the limits for the precipitation of the antitoxin-bearing proteins. Thus, in plasma of which the reaction lay between P_H7·4 and 8·3 the main part of the antitoxin-bearing proteins was precipitated in the

Fig. 7. Showing the relationship between the precipitation of the antitoxin and of the serum proteins from cresylised plasma B by sodium sulphate



The circumscribed points ⊙ represent the data for the unheated plasma.
The points at the intersection of two lines × represent the data for the heat-denaturated plasma
The figures by the side of each point indicate the concentration of sodium sulphate in the plasma

fraction isolated between 12 and 18·5 per cent. of sodium sulphate: from the same plasma to which 0·30 per cent. of cresylic acid had been added, the precipitation limits were respectively reduced to 11·5 and 16·5 per cent. of the sulphate[1]. Furthermore, in the former case the limits were raised further by the dilution of the plasma; in the latter case they were lowered by an increase in the concentration of the cresylic acid in the plasma.

[1] A similar lowering of the precipitation limits was seen in the precipitation of cresylised plasma by ammonium sulphate.

In the *heated* plasmas the precipitation limits with sodium sulphate were considerably lower than those given above for the unheated liquids, and, as was anticipated, the increased precipitability of the antitoxin proved to be a function of the heat-denaturation of the antitoxin-bearing proteins.

The data furnished from the study of the precipitation of the cresylised plasma B by sodium sulphate have been embodied in the curve in Fig. 7 in which the circumscribed points and those at the junction of a cross respectively denote the values obtained from the unheated and the heated liquids.

A study of the curve shows that, in the *unheated* cresylised plasma, only a small amount of antitoxin (less than 5 per cent.) was precipitated in the pseudoglobulin-euglobulin zone, viz. at concentrations from 9—11·5 per cent. Beyond this stage the points representing the relative precipitation of antitoxin and protein lie on a straight line the slope of which remains constant until the whole of the antitoxin has been precipitated.

In the *heated* cresylised plasma there was an increased precipitation of protein and of antitoxin at each of the stages investigated. Even at a concentration of 8 per cent. of sodium sulphate there was an appreciable precipitation of denaturated pseudoglobulin and its associated antitoxin; the precipitation of antitoxin and pseudoglobulin was complete on the addition of 15·5 per cent. of sodium sulphate. The position of the corresponding points on the curve shows that, in heated plasma also, there is a direct proportionality between the precipitation of antitoxin with the protein at each stage with the sulphate, and, furthermore, that the points representing this relationship fall on the same straight line as that connecting the points obtained for the unheated liquids.

The gravimetric estimations of the relative amounts of the individual proteins precipitated at various stages with the sulphate indicated that the precipitation of antitoxin from the plasma coincided with that of the pseudoglobulin.

Calculations were accordingly made of the percentages of the total antitoxin and of the total pseudoglobulin precipitated at each stage with the sulphate and the data thus obtained were incorporated in the curve in Fig. 8, the positions for the data from unheated and heated plasmas being indicated by circumscribed and by crossed points respectively.

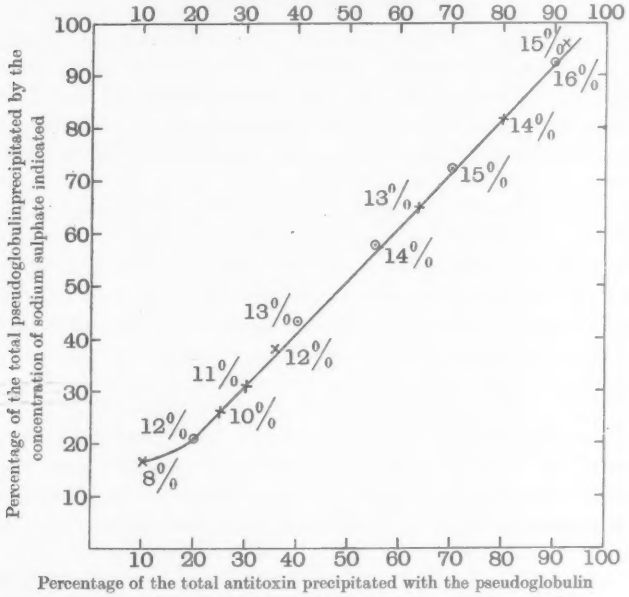
It will be seen that, in the unheated liquids, there is a comparatively small load of antitoxin associated with the fractions of the pseudoglobulin precipitated in the pseudoglobulin-euglobulin zone, and that, beyond this zone *the percentage of the total pseudoglobulin successively precipitated by increasing concentrations of the sulphate is a linear measure of the percentage of the total antitoxin associated with the precipitate.*

In the heated liquids there was no disturbance of this relationship between the relative precipitation of pseudoglobulin and antitoxin. The heat-denaturation merely served to induce the precipitation of a particular pro-

portion of the pseudoglobulin and its associated antitoxin at a concentration of sodium sulphate lower than that required for their precipitation from the unheated plasma.

These results furnish evidence in confirmation of the conclusions drawn from the investigation of the fractional precipitation of the pseudoglobulin by ammonium sulphate, viz. that from the point of view of reducing the proportion of protein associated with the antitoxins of sera, there are no practical advantages to be gained by the preliminary heat-denaturation of the serum proteins [Homer 1919].

Fig. 8. Showing the relative precipitation of antitoxin and pseudoglobulin from cresylised plasma B by sodium sulphate



The points ⊙ refer to data obtained from the precipitation of unheated plasma
The points × refer to data obtained from the precipitation of heat-denaturated plasma
The figures by the side of each point indicate the concentration of sodium sulphate used for precipitation

III. The application of the above results to the routine concentration of sera.

The question as to whether the routine concentration of antitoxic plasma shall be carried out with unheated or with heat-denaturated plasma can only be decided after a careful comparison of the respective end products as regards (*a*) their ease of filtration through Berkfeld and through white Doulton filter candles, and (*b*) the clinical after-effects of their administration to the animal or to the human.

Unfortunately, through the lack of the necessary training, I cannot produce evidence on the latter point other than that referred to in a previous note [Homer 1918, 2] in which attention is drawn to variations in the toxicity of cresylic acid in plasma according as the serum proteins in the latter have been subjected to heat-denaturation or not.

The conditions regulating the preparation of satisfactory end products from the ammonium sulphate or from the sodium sulphate fractionation of unheated and of heat-denaturated plasma have been studied separately and the following observations have been made.

(i) *The filtration of the end products from the concentration of* unheated *plasma.*

In order to obtain clear end products it is essential to fix the precipitation limits with ammonium sulphate or with sodium sulphate so as to ensure the elimination of euglobulin. Satisfactory products are obtained from the dialysis of the protein fractions isolated from unheated plasma (P_H 7·4—8·3) between concentrations of ammonium sulphate of which the lower limit is not less than 34 per cent. of saturation. In the cases where sodium sulphate is used the lower limit should not be less than 11·5 per cent.

Where the lower limit for the precipitation with ammonium sulphate was 33 per cent. of saturation or less, or was less than 11·5 per cent. with sodium sulphate, the end products were opalescent and filtered badly; the filter candles during the filtration became coated with a slimy deposit of euglobulin which retarded and ultimately stopped the process.

The Second Fraction precipitates isolated from unheated plasma (P_H 8·3) between 34 and 46 per cent. of saturation with ammonium sulphate or between concentrations of 12 and 18·5 per cent. of sodium sulphate contained the bulk of the antitoxin. The corresponding precipitates from cresylised plasma (0·30 per cent.) contained a lower proportion of the antitoxin for the reason that the presence of the cresylic acid induced an increased precipitation of the antitoxin-bearing proteins with the First Fraction precipitates.

The antitoxin carried down with the First Fraction whether precipitated by sodium sulphate or by ammonium sulphate, was, in all cases, recovered by extraction of the precipitates with a saturated solution of sodium chloride.

Contrary to the experience with heat-denaturated plasma it was found that the end products from the fractionation of unheated cresylised plasma between the above limits with ammonium sulphate were opalescent and filtered less readily than those obtained from the non-cresylised plasma. Further experiment showed that in the cresylised plasma the concentration of the precipitating sulphate required to ensure the agglutination and filtration of the cresylic acid-protein complex with the First Fraction precipitates was greater than that required for the complete precipitation of euglobulin.

The same phenomena were exhibited to a less marked degree in the precipitation of cresylised plasma by sodium sulphate.

These results indicate that, in the concentration of antitoxic plasma by the fractional precipitation of the unheated plasma, the preliminary addition of cresylic acid is disadvantageous. For, not only does it reduce the proportion of antitoxin associated with the Second Fraction precipitates, but it also leads to the production of less readily filterable end products than are furnished from the similar treatment of non-cresylised plasma.

(ii) *The filtration of the end products from* heat-denaturated *plasma.*

In previous communications I have described techniques [Homer 1917, 1918, 3] which gave the best results for the fractional precipitation of heat-denaturated plasma by ammonium sulphate. I am still convinced that more consistent and more reliable results are to be obtained from the preliminary adjustment of the reaction than from the alternative procedure of adding 0·30 per cent. of cresylic acid to the plasma.

In the present investigation also it has been found desirable to regulate the heat-denaturation of the serum proteins prior to their fractional precipitation with sodium sulphate.

The directions previously given for the suitable adjustment of the reaction of the plasma may be somewhat simplified. Satisfactory values for the heat-denaturation are obtained by the adjustment of the reaction of the plasma, so that 10 c.c. show a very faint green tinge with α-naphthol-phthalein; the colour change is readily detected when the plasma is viewed slantwise.

A further simplification of the routine technique in regard to the heating processes hitherto advocated in order to ensure the required heat-denaturation and the subsequent separation of heat-denaturated protein with the First Fraction precipitate can be effected as follows. To the adjusted plasma, prior to its being heated, is added the amount of sodium sulphate or ammonium sulphate required for the precipitation of the First Fraction precipitates. The sulphated mixtures are heated to 58° and kept at that temperature for four to five hours. They are then filtered and from the combined volume of the filtrates and washings are precipitated the Second Fraction precipitates in the usual way. By this method of procedure the two stages of the heating process are reduced to one.

However, in the case of the ammonium sulphate precipitates the advantage thus gained by combining the two stages of the heating is minimised by the greater losses of antitoxin subsequently experienced; the latter are no doubt due to the hydrolysis of ammonium sulphate and its dissociation into its component parts during the prolonged heating.

On the other hand, as sodium sulphate is the salt of a strong acid and a strong base, no such secondary complications arise.

Presumably this difference in the nature of the two sulphates also accounts for the somewhat sharper line of demarcation shown in the precipitation of eu- and pseudo-globulin from plasma by sodium sulphate than when

ammonium sulphate is employed; a phenomenon which favours the use of the former sulphate in the concentration of antitoxic sera.

In the heated plasmas containing sodium sulphate, in which the heat-denaturation had been regulated as above, the precipitation of the euglobulin was complete at a concentration of 8 per cent. of anhydrous sodium sulphate, but the separation of the precipitated protein by filtration through paper was impossible. Just as with ammonium sulphate, the indifferent filtration of the First Fraction mixtures was due to the presence of incompletely agglutinated particles of heat-denaturated protein, and unless the separation of the latter was ensured at this stage, the filtration of the end products was impossible. For this purpose it was found necessary to increase the concentration of sodium sulphate to 10 per cent., but, in order to guarantee the rapid filtration of the First Fraction precipitates it was advisable to use 11 per cent. of the sulphate, a procedure which entailed the precipitation of at least 35 per cent. of the antitoxin with the protein eliminated at this stage. However, provided that the heat-denaturation has not been greater than that recommended for the routine work, the antitoxin thus precipitated with the First Fraction precipitates can always be recovered by the extraction of the precipitate with a saturated solution of salt.

Those engaged in the routine work must be prepared to find that the precipitation limits discussed above may not give quite the same results when applied to the concentration processes carried out in their laboratories. There are many conditions which affect the precipitability of the serum proteins, and in the routine work the important factor, dilution, is apt to be forgotten. Thus, after the removal of the First Fraction, the solution contains considerably less protein than the original plasma; the protein content is further reduced by the addition of the filtered washings, a procedure which tends to modify the precipitation limits for the proteins (*ante*, p. 284). The investigation of practical details of this nature are best carried out by the individual worker under the conditions which prevail in his particular laboratory.

Summary.

The results of the investigation have shown that:

(1) In the precipitation of the serum proteins by sodium sulphate there are no critical points marking the limits for the precipitation of the individual proteins.

(2) The concentration of either ammonium sulphate or sodium sulphate required for the precipitation of the serum proteins is affected by the reaction and by the dilution of the plasma and by the addition of cresylic acid to the plasma. In the latter case the extent of the increase is a function of the concentration of cresylic acid in the plasma.

(3) Within the limits of the error of the experiment, the respective decreases in the molecular concentration of sodium sulphate and of am-

monium sulphate measured under the conditions given in (2) are practically identical.

(4) The percentage precipitation of the antitoxin with the proteins precipitated at various concentrations of sodium sulphate is a linear measure of the percentage precipitation of the antitoxin-bearing proteins. This relationship is undisturbed by the heat-denaturation of the serum proteins induced during the heating of adjusted plasma at 58° for four to five hours.

(5) In the concentration of antitoxic plasma by its fractional precipitation with sodium sulphate or ammonium sulphate, results can be obtained by the suitable fractionation of the unheated plasma similar to those which have hitherto been obtained with heated plasma.

(6) While the addition of cresylic acid materially aids the concentration of heat-denaturated plasma, its use with unheated plasma is to be deprecated.

(7) The agglutination of the particles of precipitated protein in the eu- and pseudo-globulin zone seems to be more satisfactory with sodium sulphate than with ammonium sulphate.

(8) As sodium sulphate, in contradistinction to ammonium sulphate, does not hydrolyse in solution, the sodium-sulphate-plasma First Fraction mixtures can be heated for four to five hours at 58° without loss of antitoxin. This method of procedure favours the production of clearer end products than would result from conducting the heating in the two stages adopted in the ammonium sulphate method.

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XXX. THE DIRECT REPLACEMENT OF GLYCEROL IN FATS BY HIGHER POLYHYDRIC ALCOHOLS. PART I. INTERACTION OF OLEIN AND STEARIN WITH MANNITOL.

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(*Received August 18th, 1919.*)

Glycerol is required in abnormally large quantities during war and therefore just at such times as a shortage may be expected of oils and fats which are the essential raw materials in the manufacture of glycerol as at present practised. As the by-products in the manufacture have but a limited application as food-stuffs, various suggestions have been made with a view to extending this application and, among others, that of converting the fatty acids into ethyl and other esters has been considered. It is but a short step from this to the idea of esterifying the acids with polyhydric alcohols similar in type to glycerol and this again suggests the possibility of using sugars and their simpler derivatives for a like purpose. The latter line of inquiry appears especially promising inasmuch as many of the substances in this group are themselves valuable as food-stuffs.

We have been associated with Professor R. Robinson in considering the chemical aspects of such problems and in consultation with him decided to attempt a more direct solution than esterification of the preformed fatty acids.

It is well known that small quantities of acids or alkalis facilitate the exchange of alcohol residues in esters and accelerate the attainment of equilibrium in the resulting system. In those instances in which one of the products can be removed from the sphere of action a complete conversion of one ester into another may be effected.

It was anticipated that by bringing together, in presence of an acid or alkaline catalyst, a natural oil or fat and a polyhydric alcohol having a higher boiling point than glycerol the latter might be removed by distillation under reduced pressure.

Our first experiments were made with mannitol as the polyhydric alcohol and the catalyst used was sodium ethoxide, the alcohol arising therefrom being carried over with the glycerol formed during the reaction. The results justified our anticipations and although the products are not simple esters

of mannitol itself as we at first supposed, they resemble true glycerol fats in appearance and to some extent in taste and are reported by Professor W. D. Halliburton and Dr Drummond [1919] to be assimilated by rats to much the same extent as is olive oil.

Interaction of olive oil with mannitol. Formation of "Mannitol olive oil."

The olive oil used was a good commercial sample and had an acid value corresponding with 2·5 per cent. of free oleic acid.

100 g. of this oil was mixed in a Claisen distillation flask with 31 g. of pure mannitol and 1·5—2 per cent. of sodium ethoxide (freshly prepared by evaporating a solution of sodium in alcohol and heating gently until the mass appeared dry). One thermometer was kept with its bulb immersed in the mixture itself (the temperatures recorded by this are indicated hereafter by the letter T) and another with its bulb immersed in the issuing vapours in the exit tube of the flask (temperatures indicated by the letter t).

Experience having shown that on the small scale no harm resulted therefrom, the heating was usually carried out for convenience by direct contact with the flame of a Bunsen burner. Alcohol in small quantities appeared to be evolved first ($T = 87$—$90°$, $t = 25$—$30°$); later the mannitol crystals melted and two fluid layers were formed in the flask ($T = 140°$) while considerable frothing ensued which continued as T rose to 215°. Here frothing subsided to some extent and as the heating went on the two fluid layers began to react, the upper one becoming turbid, and appearing to boil. Water, contaminated with a little oily or solid matter, distilled at this point ($t = 54°$) and continued to do so as the temperature rose. A steady bubbling now took place (T steady at 236—240° during 10—15 minutes; t about 52°), and the lower layer decreased in bulk. With further heating a thick fluid began to distil (t rose to 170° while T was raised to 270°) and the two layers in the flask merged into one. The heating was not pressed much further as some darkening in the product occurred if T was allowed to rise to 275° and very little more distillate was obtained.

Examination of distillate. This formed a thick viscid fluid on which floated a little oil; it was diluted with water, extracted with ether to free it from oily impurities and the aqueous solution was evaporated on the steam bath. A thick, viscid, sweet liquid remained (10 g.) which on distillation yielded 8 g. of a thick, almost colourless fluid, boiling at 179° at 14 mm. pressure. This was practically pure glycerol and was identified by conversion into the crystalline benzoate. As the amount of glycerol theoretically obtainable from the quantity of olive oil used is 10·4 g. it is evident that the yield was excellent in spite of losses inevitable in the manipulation of such comparatively small quantities of material.

Examination of residue. The fluid remaining in the flask was dissolved in ether and shaken with water, the whole being treated drop by drop with

acetic acid until a permanent slight acidity towards litmus was attained. The ethereal solution was separated and shaken several times with water to remove sodium salts together with any glycerol and mannitol or its anhydrides which might be present, though in point of fact when the foregoing procedure was followed the aqueous fluids yielded only very small quantities of residue, mainly sodium acetate, so that practically no free glycerol or mannitol was present.

After a preliminary drying over sodium sulphate and evaporation of the ether, the residual oil was more fully dried by heating it in a vacuum at 200—220° for several hours while a continuous stream of air was passed through it.

The product is an oil with a brownish yellow colour and a very faint odour. In taste and fluidity it is not dissimilar to olive oil. When kept for some weeks it gradually becomes somewhat cloudy and this effect appears to be due to deposition of a fine powder (stearates?) which may be removed by filtration. Professor Halliburton kindly undertook the examination of the properties of this oil when used as a food and a report on the subject appears elsewhere in this *Journal* [Halliburton and Drummond, 1919].

We have formed definite views on the chemical character of the oil only after consideration not only of its percentage composition and the amount of free and combined acid, but also of free hydroxyl, which was determined by means of magnesium methyl iodide in amyl ether.

By the interaction of mannitol and glyceryl oleate there may be formed a number of oleates in which from one to six of the hydroxyls of mannitol are esterified, as well as a series of wholly or partially esterified derivatives of mannitan and of isomannide, compounds which are derived from mannitol by loss of one and two molecules of water respectively. The percentages of carbon and hydrogen in many of these esters hardly admit of their being used to distinguish the esters from one another in a mixture, even if the entire absence of any esters of glycerol could be regarded as quite certain. On the other hand the percentages of combined acid, calculated as oleic acid, and especially of the free hydroxyl in the several possible esters vary very considerably as is shown in the following table from which some esters have been omitted because their composition differs so widely from that of the "mannitol olive oil" that they could only be present there if at all in quite small proportions.

While it seems possible that several or all of the above esters are present to some small extent in "mannitol olive oil" a careful examination of the table will show that in every instance but two there is at least one characteristic figure for each which differs widely from the one found experimentally. The two exceptions are the di-oleates of mannitan and isomannide.

Compositions of various esters of Oleic Acid.

Compound	Mol. wt. and formula	% OH	% combined acid	% C	% H
(1) Mannitol penta-oleate	$C_{96}H_{174}O_{11}$ (1502)	1·13	93·8	76·69	11·58
(2) Mannitol tetra-oleate	$C_{78}H_{142}O_{10}$ (1238)	2·7	91·1	75·6	11·47
(3) Mannitol tri-oleate	$C_{60}H_{110}O_9$ (974)	5·22	86·85	73·92	11·29
(4) Mannitol di-oleate	$C_{42}H_{78}O_8$ (710)	9·57	79·43	70·98	10·98
(5) Mannitan tri-oleate	$C_{60}H_{108}O_8$ (956)	1·77	88·49	75·31	11·29
(6) Mannitan di-oleate	$C_{42}H_{76}O_7$ (692)	4·91	81·5	72·83	10·98
(7) Isomannide di-oleate	$C_{42}H_{74}O_6$ (674)	0	83·68	74·77	10·97
(8) Isomannide mono-oleate	$C_{24}H_{42}O_5$ (410)	4·01	68·78	70·24	10·24
Olive oil	$C_{57}H_{104}O_6$ (884)	0	95·7	77·36	11·76
Found in "mannitol olive oil"	—	2·17	83·65	72·82	10·77

Interaction of mannitol with stearin. Formation of "Mannitol stearin."

Mannitol and stearin were mixed and heated in a vacuum with a little sodium ethoxide under much the same conditions as in the experiments where olive oil instead of stearin was used. The non-volatile residue was dissolved in chloroform, in which it dissolved more freely than in ether, and the resulting solution was treated with acetic acid and water as described in the preceding experiments. The washed and dried product was solid at ordinary temperatures. It was easily soluble in cold chloroform, hot petroleum (B.P. 80—100°), hot ethyl acetate, acetone or benzene. It dissolved in hot alcohol but was nearly insoluble in the cold. It separated from acetone on cooling as a granular powder melting at 67—72°.

Advantage was taken of the solid character of this product to do what was not possible in the case of the material from olive oil, namely to separate it into two main fractions by crystallisation from acetone. It was anticipated that if there were present any appreciable quantities of impurities markedly different in character from the main mass, evidence of this would be obtained by analysis of different fractions. In point of fact however no appreciable difference was found.

It is also interesting to observe that if the molecular proportion of mannitol to stearin used in the original reaction is increased from 1·5 to 2·25, the product is not appreciably altered in percentage composition and it seems probable that any excess of mannitol passes over with the glycerol during the heating with stearin in the first process or is washed out afterwards.

In the following table are exhibited the four characteristic data for the series of stearic esters derived from mannitol and its dehydration products.

Compositions of various esters of Stearic Acid.

Compound	Mol. wt. and formula	% OH	% combined acid	% C	% H
(1) Mannitol penta-stearate	$C_{96}H_{184}O_{11}$ (1512)	1·12	93·91	76·19	12·16
(2) Mannitol tetra-stearate	$C_{78}H_{150}O_{10}$ (1246)	2·72	91·1	75·12	12·03
(3) Mannitol tri-stearate	$C_{60}H_{116}O_9$ (980)	5·2	86·93	73·47	11·83
(4) Mannitol di-stearate	$C_{42}H_{82}O_8$ (714)	9·52	79·55	70·58	11·48
(5) Mannitan tri-stearate	$C_{60}H_{114}O_8$ (962)	1·76	88·56	74·83	11·85
(6) Mannitan di-stearate	$C_{42}H_{80}O_7$ (696)	4·88	81·6	72·41	11·49
(7) Isomannide di-stearate	$C_{42}H_{78}O_6$ (678)	0	83·9	74·32	11·50
(8) Isomannide mono-stearate	$C_{24}H_{44}O_5$ (412)	4·12	68·93	69·9	10·67
Stearin	$C_{57}H_{110}O_6$ (890)	0	95·73	76·85	12·36
Found in "mannitol stearin"	—	2·2	82·44	73·1	11·26

What was true of the original "mannitol olive oil" is equally true, *mutatis mutandis*, of "mannitol stearin," which from the above therefore appears to consist mainly of the di-stearates of mannitan and isomannide. We have to record with regret that we have not yet been able to obtain direct evidence of the presence of mannitan and isomannide residues in either product and feel doubtful whether an examination of the products of hydrolysis would lead to very conclusive results.

Summary.

1. By distillation of olein or stearin with mannitol under reduced pressure in presence of a little sodium ethoxide, nearly the whole of the glycerol present in the original fatty compound is expelled.

2. The maximum yield of glycerol is reached when the proportion of fat to mannitol corresponds with two molecules of fat to three of mannitol.

3. The other main products are in both cases water with a little alcohol and a substance which is similar in many properties to the original fat.

4. The composition of the product, whether made with the above proportions of fat and mannitol or with excess of mannitol, corresponds with that of a mixture of the di-oleates (or di-stearates) of mannitan and isomannide. This closely corresponds with the observations mentioned under (2) above.

Professor J. C. Irvine has very kindly confirmed the description we submitted to him of our original mode of preparing glycerol and "mannitol olein" from olive oil. The extension of the work with sugars (and their derivatives other than mannitol) has been left in his hands.

This investigation was carried out for the Food Investigation Board (Dept. of Scientific and Industrial Research) who defrayed the expenses involved.

Reference.

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XXXI. THE DIRECT REPLACEMENT OF GLYCEROL IN FATS BY HIGHER POLYHYDRIC ALCOHOLS. PART II. THE VALUE OF SYNTHETIC MANNITOL OLIVE OIL AS A FOOD.

By

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(*Received August 18th, 1919.*)

In the preceding communication Lapworth and Pearson [1919] have described the preparation of a synthetic oil by replacing glycerol in olive oil by mannitol. As part of the general enquiry it was also necessary to ascertain what value, if any, this synthetic product possesses as a food-stuff. A sample of the oil was therefore submitted to us for the purpose of making feeding tests. The product supplied was a light greenish brown oil of somewhat dirty appearance, containing a small amount of solid matter in suspension. The amount of this solid matter increased when the oil was kept standing during the cold weather, and from a cursory examination it appeared to consist of the higher melting point esters (stearin). The oil possessed a taste and odour reminiscent of olive oil, but the former was if anything less pleasant than that of the natural oil.

The following data were obtained on analysis, and for comparative purposes the figures obtained on the analysis of a good quality commercial olive oil are also given. The latter was not, however, identical with the olive oil from which the synthetic oil was prepared.

	"Mannitol olive oil"	Olive oil
Free fatty acids, calculated as oleic acid	4·0 %	2·7 %
Saponification value	159	193
Iodine value	82·4	84·7

The investigation of the food value of the mannitol oil was planned to determine first, whether the oil could be assimilated by the mammalian digestive system, and secondly, whether the oil could be administered with safety over considerable periods without any deleterious influence on health. Unfortunately, the amount of material at our disposal was small, and we were obliged to abandon the idea of experiment on man or the higher animals. Such experiments would of necessity have been of very short duration and

quite unsuited to the purpose of the investigation. Accordingly we confined our attention to a study of the absorption of the oil in the digestive tract of the rat, an animal whose suitability for prolonged tests of this type is unquestioned.

As far as we have been able to ascertain, Bloor is the only investigator who has studied the digestibility of mannitol esters of the higher fatty acids in the animal body. He prepared mannide distearate and mannitan distearate and demonstrated that they were hydrolysed by the lipolytic enzymes of the pancreas [1912, 1]. He also carried out feeding experiments of short duration on cats and established the fact that the esters were absorbed from the intestinal tract. One cat received a daily ration of 3·8 g. of mannitan distearate in 5 g. of cotton-seed oil for a period of six days, and showed an absorption of 42·3 per cent. A higher absorption (53·6 per cent.) was observed in an experiment of one day when a much larger amount of the ester was administered. Experiments with isomannide distearate (M.P. 61·5°) showed that approximately 72 per cent. was absorbed, a utilisation comparable with that recorded for tristearin, a natural fat with a melting point of the same order [Arnschink, 1890]. Even better absorbed was isomannide dilaurate [Bloor, 1912, 2], for which coefficients as high as 95—97 per cent. were obtained.

Feeding Tests with "Mannitol Olive Oil" on Rats.

To make the tests of the edibility of the mannitol oil as severe as possible it was decided to administer it as the sole source of fat in the diets of the experimental animals. Such a decision introduced a disturbing element into the experiments, for it would necessitate the animals being deprived of the fat-soluble accessory factor, which for several reasons we knew must be absent from the synthetic oil. It was hoped, however, to surmount this difficulty as well as possible by employing animals which were nearly mature. Such animals may live without any apparent disturbance of health upon a diet deficient in fat-soluble A for several weeks [Drummond, 1919]. Two pairs of healthy young adult male rats were selected and confined in suitable metabolism cages. For a preliminary period both pairs were fed upon an artificial diet containing butter fat, after which they received diets containing either olive oil itself or the synthetic mannitol oil for further periods, usually of four days each.

The dietaries were compounded as follows:

	A	B	C	
Purified caseinogen	20	20	20	parts
,, starch	50	50	50	,,
Yeast extract	5	5	5	,,
Salt mixture	5	5	5	,,
Orange juice	5	5	5	,,
Butter fat	20	—	—	,,
Olive oil	10	30	—	,,
Mannitol oil	—	—	30	,,

These diets were stiff pastes which were not readily scattered about by the animals during feeding. Every morning fresh water and a ration of diet were placed in the food receptacles in the cages, and the unconsumed diet from the previous twenty-four hours was taken out and weighed. The nitrogen balance was determined in order to follow the general nutritive condition of the animals. Fat was estimated in the food mixtures and fæces by prolonged ether extraction in a Soxhlet apparatus, after dehydration at 110° for twenty-four hours. It was originally proposed to attempt an estimation of the unabsorbed mannitol oil by a polarimetric method, as was done by Bloor [1912, 2], but it was ascertained that the low specific rotation of the oil ($[\alpha]_D^{20} = -1{\cdot}44°$) rendered such a procedure out of the question.

Tables I and II contain the detailed experimental results. The animals all tended to lose weight when receiving the mannitol oil diet, and their food consumption was not good. Rats 3 and 4 both appeared in somewhat poor condition at the termination of their period of 11 days on the mannitol diet. In all cases, however, the absorption of the mannitol oil was good. From the various utilisation values recorded the following approximate averages have been obtained.

	Percentage utilisation
Butter fat and olive oil	97·8
Olive oil	96·6
"Mannitol" olive oil	95·8

From the point of view of the edibility of the mannitol oil it was important to determine whether the slight decline in health which was apparent in the experimental animals on the mannitol oil dietaries was due to some deleterious action of the oil *per se*, or to the fact that they were being maintained on a ration deficient in one of the indispensable accessory factors. To test this point a batch of young rats of uniform age and sex were selected and divided into two sets. One of these received a diet containing mannitol oil together with butter fat as a source of fat-soluble A (Diet E), the other received a diet containing equivalent amounts of natural olive oil and butter fat (Diet F). The composition of the two rations was as given below:

	Diet E	Diet F
Purified caseinogen	20 parts	20 parts
,, starch	50 ,,	50 ,,
Yeast extract	5 ,,	5 ,,
Orange juice	5 ,,	5 ,,
Salt mixture	5 ,,	5 ,,
Butter fat	10 ,,	10 ,,
Olive oil	— ,,	10 ,,
Mannitol oil	10 ,,	— ,,

Unfortunately the experiments were restricted by the small amount of mannitol oil which was available. The results which were obtained are tabulated in Table III.

Table I.

Day	Remarks	Body wt. g. Rat 1	Body wt. g. Rat 2	Rectal temp. °F. Rat 1	Rectal temp. °F. Rat 2	Food eaten g.	% Fat in food	Total fat consumed g.	Fat in faeces g.	Fat absorbed g.	% Utilisation	N in food g.	N in urine g.	N in faeces g.	N balance g.
1	Butter Period	164	160	100·3	100·2	16·9									
2	(Diet A)					13·3	32·40	16·30	0·38	15·92	97·7	1·19	0·81	0·10	+0·28
3						20·1									
4	Olive Oil Period	158	154	100·2	99·6	21·1									
5	(Diet B)					21·2	23·68	20·00	0·49	19·51	97·5	1·83	0·96	0·14	+0·73
6						21·5									
7						20·5									
8	Olive Oil Period	178	172	100·2	99·7	18·6									
9	(Diet B)					15·0									
10						15·3	23·68	13·24	0·54	12·70	95·9	1·02	0·80	0·12	+0·10
11						7·0									
12	Mannitol Oil Period	171	171	—	—	13·6									
13	(Diet C)					17·6									
14						16·0	30·10	19·32	0·63	18·69	96·7	1·73	1·46	0·16	+0·11
15						17·0									
16	Mannitol Oil Period	171	171	100·8	102·8	7·9									
17	(Diet C)					12·0									
18						15·2	30·10	14·85	0·92	13·93	93·8	1·29	1·24	0·20	−0·15
19						14·2									
20		167	158	—	—	0									
21	Butter Period					20·1									
22	(Diet A)					20·5									
23						13·8	32·20	24·20	0·46	23·74	98·1	1·93	1·46	0·09	+0·38
24						20·8									
25		176	172	100·2	101·6	0									

Table II.

Day	Remarks	Body wt. g. Rat 3	Body wt. g. Rat 4	Rectal temp. °F. Rat 3	Rectal temp. °F. Rat 4	Food eaten g.	% Fat in food	Total fat consumed g.	Fat in faeces g.	Fat absorbed g.	% Utilisation	N in food g.	N in urine g.	N in faeces g.	N balance g.
1	Butter Period	154	148	101·0	101·1	15·5									
2	(Diet A)					16·7	32·4	16·95	0·41	16·54	97·6	1·24	0·76	0·07	+0·41
3						20·1									
4	Mannitol Oil Period	171	161	—	—	25·8									
5	(Diet C)					15·6									
6						17·9	26·0	18·60	0·37	18·23	98·1	2·01	1·04	0·07	+0·90
7						12·2									
8	Mannitol Oil Period	163	157	100·2	101·8	13·6									
9	(Diet C)					13·0									
10						13·9	26·0	13·70	0·78	12·92	94·3	1·48	1·22	0·08	+0·18
11						12·2									
12	Mannitol Oil Period	160	152	99·6	99·2	9·2									
13	(Diet C)					11·3									
14						9·2	30·1	10·99	0·32	10·67	97·1	0·94	1·06	0·09	−0·21
15						6·8									

Table III. *Average body weight in grams of two groups of six male rats.*

Days	Diet E Mannitol oil-butter diet	Diet F Olive oil-butter diet
0	64	67
7	72	85
14	94	98
21	106	112
28	121	132
35	136	148
42	153	162

On the whole, the growth of the rats which received the butter-fat-olive-oil mixture was more satisfactory than that of the animals on the mannitol oil diet. The difference in the final weights is not very marked, and it is difficult to express an opinion as to whether it is of any significance, particularly in view of the small number of animals employed.

At the conclusion of the experiment the mannitol-fed animals appeared in quite as good condition as the control lot, so that it appears safe to assume that no toxic factor was present in the sample of synthetic mannitol olive oil. That this oil was less palatable than olive oil appears likely, however, from the fact that the food consumption of the batch on Diet E was appreciably less than of that receiving Diet F.

The importance that was attached to this investigation in the period during the recent war, when urgent demands of fat for glycerol were bringing about a drastic reduction in the amount available for edible purposes, is fortunately now no longer so great. The results are therefore placed on record purely in the light of their scientific interest.

Summary.

1. A synthetic oil prepared by replacing glycerol in olive oil by mannitol is utilised by the animal organism to practically the same extent as olive oil itself.

2. No toxic action was found to follow the prolonged administration of this oil to rats.

This investigation was carried out for the Food Investigation Board (Dept. of Scientific and Industrial Research), and the expenses involved were defrayed out of a grant from the Royal Society.

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XXXII. RELATIVE ANTI-SCORBUTIC VALUE OF FRESH, DRIED AND HEATED COW'S MILK.

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(1) Experiments with guinea-pigs by R. E. Barnes.
(2) Experiments with monkeys by E. M. Hume.

Introduction.

In two previous communications [Chick, Hume and Skelton 1918, 1, 2] one of us (E. M. H.), in conjunction with other workers in this Department, has shown that raw cow's milk must be classed among the less valuable foodstuffs as regards anti-scorbutic properties. It was shown, for example, that whereas in case of many raw fruits and vegetables amounts varying from 1·5 to 10 g. daily will protect guinea-pigs from scurvy upon a diet otherwise devoid of all anti-scorbutic material, 100—150 cc. of raw cow's milk was needed to give similar protection. The opinion was also put forward that dried milk was even less valuable than raw milk in respect of anti-scorbutic properties; this decision was reached mainly on theoretical grounds—there being at that time few experimental results available. But it had been shown that sundry other foodstuffs, rich in the anti-scorbutic vitamine, lose the greater part of this principle when no longer associated with a living tissue, as, for example, when the foodstuff is dried or preserved by other means. As regards dried milk, this view is in opposition to that expressed by many medical authorities, who, on the basis of their clinical experience, consider that dried milk is in every respect a satisfactory substitute for fresh milk and may be adopted as a sole diet for children without any risk from scurvy [see Coutts 1918, Naish 1914].

The present paper deals with an attempt to gain by direct experiment a quantitative estimate of the anti-scorbutic value of dried milk compared with raw milk. The first series of experiments was made with guinea-pigs by one of us (R. E. B.) and the details of the work are set out in the next chapter.

The methods and technique were the same as those employed in the previous work already mentioned. Guinea-pigs are however not well suited for work upon the anti-scorbutic value of milk. In order to maintain health

and to prevent scurvy, these animals need a comparatively large amount of anti-scorbutic material in their diet; in case, therefore, of a foodstuff like milk with a low content of anti-scurvy vitamine, it is necessary for large quantities to be consumed. It is against the habit and nature of these animals to take much liquid and we have never come across an animal which would take voluntarily the large daily ration (100 cc. and upwards) of raw milk necessary to afford protection from scurvy. Hand feeding of these large quantities is indescribably tedious and in many cases they cannot be tolerated without digestive disturbance. The results obtained in a few successful cases out of a large number of trials are given below (Table I), together with those obtained by similar methods with dried milk. The inferiority of the latter as regards anti-scorbutic properties is clearly shown.

The importance of this result in relation to the artificial feeding of infants made it very desirable to repeat the experiments with a second experimental animal. Young, growing monkeys were selected, and the work was carried out by E. M. H. Many monkeys are extremely fond of milk in any form, and once the right species and individual have been selected, there is little difficulty in persuading the animal to take the daily ration ordained. Symptoms of scurvy in the monkey are more closely allied to those of the human disease than is the case with guinea-pig scurvy, and in these young monkeys appeared to be identical with those of infantile scurvy. The results obtained were in complete accord with those obtained from the work with guinea-pigs. The quantity of milk which, added to a diet containing no other anti-scorbutic material, successfully prevented the onset of scurvy when given in the raw condition, failed to avert it when prepared from dried milk; and, further, the scurvy symptoms developed in the latter case could be cured if the dried milk ration was substituted by an equal amount of raw milk.

In these experiments with monkeys, evidence was obtained that the anti-scorbutic value of cow's milk (and of the dried milk prepared from it) was dependent to some degree upon the season of the year, summer milk, from May onwards, being richer in anti-scorbutic properties than winter milk (see below, p. 316). This fact is doubtless to be attributed to the difference in the cow's diet at these different seasons, the winter diet of mangolds, hay, cotton cake and cereals being poor in anti-scorbutic vitamine when compared with the summer diet of fresh greenstuff.

The bearing of these results upon the problems connected with infant feeding will be discussed later on, when the experimental work has been described in detail.

EXPERIMENTS WITH GUINEA-PIGS, BY R. E. BARNES.

Raw Milk.

The fresh milk used for these experiments and for those with monkeys, described below, was obtained from a model dairy and was very pure, specially delivered country milk.

The successful trials with raw milk are comparatively few for the reasons given above. The results of six experiments set out in Table I *a* afford, however, useful confirmation of similar work described in the previous communication referred to above [Chick, Hume and Skelton 1918, 1]. The diet, as then, consisted of oats, wheaten bran and fresh cow's milk, and it was again found that scurvy could be prevented and health maintained in those cases in which 100—150 cc. were taken.

The symptoms of guinea-pig scurvy and the post-mortem appearances have been described in previous communications [Chick, Hume and Skelton 1918, 2; Delf and Tozer 1918] and it is unnecessary to recapitulate them here.

Of these six experiments made with raw milk, the cream was removed in three cases by simple centrifuging. Milk thus separated seemed to agree with the animals better than full cream milk, and fewer animals were rejected after trial. As regards the anti-scorbutic value, no evidence was obtained that this treatment had any marked influence.

Dried Milk.

The dried milk used was a well-known commercial brand prepared by the Hatmaker process. In this process milk is poured in a thin film over steam-heated rotating cylinders, the water of the milk is rapidly evaporated and the solids left in a thin skin on the surface of the drums. After the drum has made about two-thirds of a revolution this skin is scraped off with knife blades, broken up and passed through a sieve as a finely granular powder. The temperature of the revolving drums is considerably over 100°, but the time of contact is stated by the manufacturers to be a few seconds only.

Two varieties of this dried milk were obtained for these experiments with the kind cooperation of the manufacturers. In one set of experiments the dried milk was quite fresh, being delivered at the Institute less than one week after manufacture, and used for experiment about one week later (Table I *b*). The second variety was imported from a distant colony and was at least six months old when received, and probably not less than nine months. It must therefore have been nearly one year old before the conclusion of some of the experiments (Table I *c*).

The results obtained gave no indication that the age of the product had much effect upon its anti-scorbutic value, but the experiments were not calculated to show any fine shades of difference. Against the greater age of this second sample of Colonial origin, must however be set the fact that the

Table I. *(a) Anti-scorbutic value of raw cow's milk; experiments with guinea-pigs. The basal diet consisted of* 10—20 *g. oats and wheaten bran mixed.*

No. of animal	Average amounts of milk consumed daily, in successive periods of 28 days, cc 1st	2nd	3rd	4th	Date experiment began	Length of experiment, days	Weight of animals in g. Initial	Highest reached	Final	Change %	Result
*675	91·8	76·7	—	—	Aug. 12th, 1918	46	360	446	330	− 8·3	Death from unknown cause; traces only of scurvy found post-mortem; no symptoms of scurvy during life
*676	90·1	109·9	128·2	—	Aug. 12th, 1918	91	356	583	580	+60·2	In good health all through experiment—no scurvy
*680	103·6	103·3	112·2	—	Oct. 28th, 1918	87	353	577	458	+29·7	Death from disease—no evidence of scurvy post-mortem
†670	129·8	88·3	—	—	July 10th, 1918	36	365	466	280	−23·2	Death from scurvy
†674	79·4	133	161·5	—	July 24th, 1918	89	334	543	442	+32·3	Animal in good condition. Slight signs of scurvy post-mortem
†681	98·1	119·3	134·9	147·2	Aug. 12th, 1918	111	340	474	474	+39·4	Animal showed signs of scurvy from 22nd day, improved from 44th day, recovered and enjoyed good health until end of experiment. Post-mortem —signs of old scurvy
					(b) Anti-scorbutic value of dried cow's milk, 1—2 *weeks after manufacture.*						
660	118·1	97·3	—	—	June 3rd, 1918	43	282	373	222	−21·2	Scurvy the main cause of death; slight infection as well
661	82·1	86·8	—	—	May 31st, 1918	48	278	293	176	−36·7	Death from scurvy, lesions very severe
668	98·7	78	—	—	July 2nd, 1918	32	342	406	298	−12·8	Death from severe scurvy
677	92·5	—	—	—	Aug. 18th, 1918	25	360	410	286	−20·5	Animal wretched, chloroformed—marked scurvy
683	106·4	70	—	—	Nov. 4th, 1918	30	348	396	266	−23	Death from scurvy, though lesions not very severe
					(c) Anti-scorbutic value of dried cow's milk, 6—9 *months after manufacture.*						
648	88·5	102·4	112·5	—	—	67	341	414	282	−17·3	Scurvy; signs in ribs only
649	97·5	120	93·1	—	—	71	306	400	238	−22·2	Death from scurvy
650	90·1	104·4	112·1	—	—	70	322	399	300	− 6·8	Death from chronic scurvy
651	92·7	100·1	115·1	—	—	72	318	380	239	−24·8	Wretched condition; chloroformed—distinct scurvy
653 *A*	82·	88	—	—	—	33	301	360	220	−26·9	Very ill, chloroformed—severe scurvy

* Full cream. † Separated.

Colonial cows, so the manufacturers stated, are grass fed all the year round. It is probable therefore that this Colonial dried milk had a much greater anti-scorbutic value to start with and even after six months may still have contained more than milk from stall fed cows even when freshly manufactured. Out of a total of five animals on each type of dried milk, not one escaped scurvy, although one animal in each series showed slight signs only (Nos. 648 and 683). On the whole, those receiving the older material lived longer, suggesting a greater reserve of anti-scorbutic vitamine, but the scurvy lesions, post-mortem, were correspondingly more severe.

When these results are compared with those obtained with raw milk, a great difference in the anti-scorbutic value is at once evident. In those cases where a large enough ration of raw milk was consumed, scurvy was prevented and in cases like 681 (Table I *a*) where the average amount consumed for the first 28 days of the experiment, 98 cc., was not enough to protect the animal, the larger rations consumed afterwards (119 cc. daily for a second period of 28 days and 135 and 147 cc. respectively for two succeeding similar periods) caused an improvement in the symptoms, and effected a successful cure. In case of the animals on dried milk no such effect was noticed. The amounts consumed (reckoned in terms of raw milk) were in many cases not less than those which successfully prevented scurvy in case of raw milk, and some experiments show a close parallel in the quantity actually taken. For example, No. 676 (Table I *a*) on raw milk enjoyed good health throughout while No. 650 (Table I *c*) on dried milk died from scurvy and these two animals consumed very similar amounts during the three periods of 28 days, into which the experiments were divided. In case of No. 676, these amounts were 90 cc., 110 cc. and 128 cc. and in the case of No. 650, 90 cc., 104 cc. and 112 cc. respectively for the three periods. It was usual for the appetite to fall off with onset of scurvy symptoms, and the usual increase in the amount of milk taken with increasing age of the animal, which is very noticeable in many of the raw milk experiments, is not apparent in case of dried milk.

Growth promoting properties of raw and dried milk.

The four curves in Fig. 1 show growth of four animals receiving respectively full cream raw milk (A); separated raw milk (B); freshly manufactured dried milk (C); dried milk 6—12 months old (D); normal diet of cabbage and grain for comparison (N). Those animals were selected for comparison whose consumption of milk was as nearly as possible the same. During the first 20—30 days of the experiment, the degree of growth is similar in all cases; with the onset of scurvy symptoms, there is decline and failure to grow in case of the animals receiving dried milk.

This failure is satisfactorily explained by the onset of scurvy and it is not necessary to assume that the growth promoting vitamines, water-soluble B, or fat-soluble A, have suffered serious damage in the process of drying. If

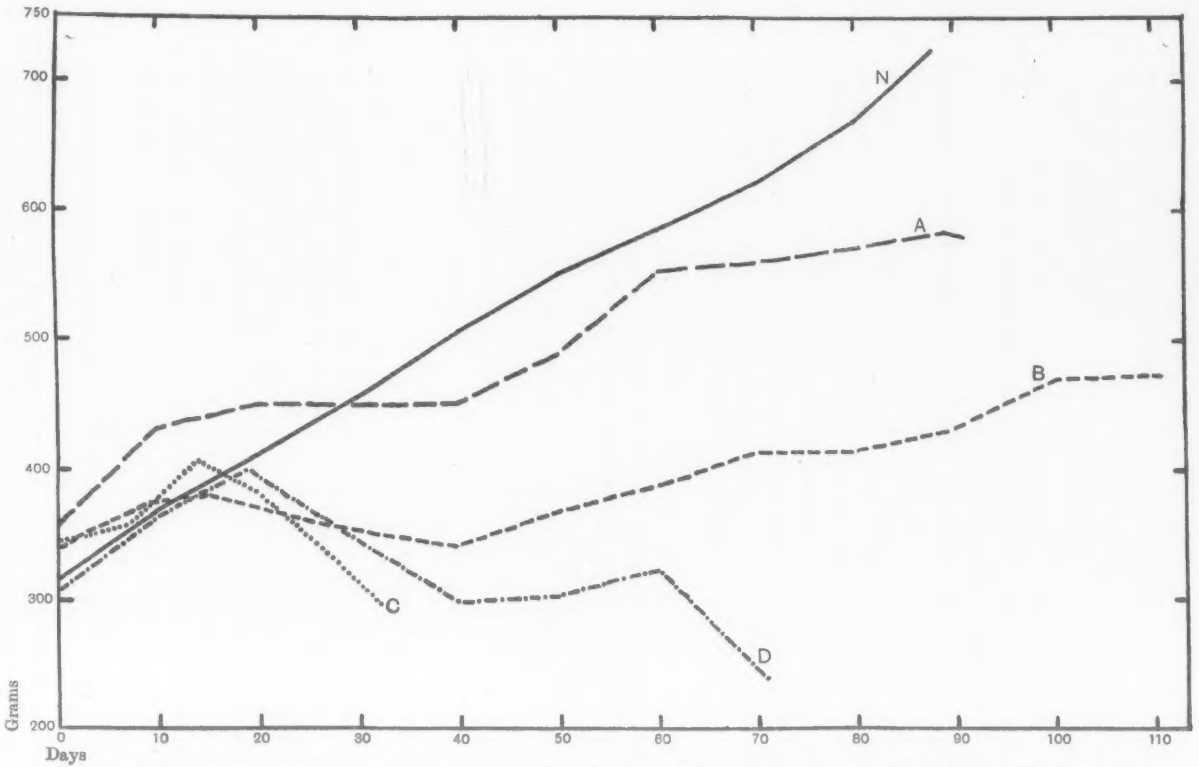

Fig. 1. Weight curves of guinea-pigs upon diet of oats, bran and milk of various types, the quantities taken being roughly equivalent.
— — — A. Full cream raw milk. No. 676. No scurvy. - - - - - B. Separated raw milk. No. 681. Slight scurvy cured later.
..... C. Dried milk (English) 1—2 weeks after manufacture. No. 668. Scurvy developed. -·-·-·- D. Dried milk (Colonial) 6—9 months after manufacture. No. 651. Scurvy developed. ——— N. Average weight curve on diet of oats, bran and cabbage leaves.

there had been any defect in the diet from this cause, the failure to maintain growth in case of dried milk would have been evident from the beginning of the experiment, whereas it was only evident after sufficient time had elapsed for the lack of anti-scorbutic material to make itself felt. It must be admitted that in case of the experiments with raw milk, owing to the size of the ration, the fat-soluble factor was probably present in large excess of the guinea-pig's requirements, and that any loss occurring during the drying might not have been sufficient to bring it below this level.

This point seemed of sufficient importance to warrant further investigation, and special experiments are now in progress to determine the relative value, as regards growth, of raw milk, dried milk and strongly heated milk respectively. In this work, the results of which will be published shortly, the quantities fed to the animals were considerably reduced, in order to determine the amounts which, in the several cases, will limit growth in guinea-pigs upon diets arranged to be satisfactory in all other respects. The results at present obtained show no significant difference between the growth promoting properties of equivalent amounts of dried and fresh milk.

Note.

While the present paper was in course of preparation two articles appeared by Hess and Unger [1919] and by Hart, Steenbock and Smith [1919] upon which comment is necessary.

Hess and Unger [1919, pp. 295, 296] find that 80 cc. daily of fresh milk or the equivalent dried by the Just-Hatmaker process are sufficient to cure guinea-pig scurvy which has already developed. This result is apparently in conflict with our experiments in which 100—150 cc. raw milk were found necessary to prevent guinea-pig scurvy and protection with dried milk was not attained at all. The reason for this discrepancy is doubtless to be found in differences in the basal diets adopted by these workers and ourselves. We employed oats and bran only, while Hess and Unger included hay *ad libitum*. Delf and Skelton [1918] have shown that vegetable leaves (cabbage) lose a great proportion, but not all, of their anti-scorbutic value when dried, and that the loss varies according to the length of time which has elapsed since the drying took place. Hay must therefore be looked upon as not entirely devoid of anti-scorbutic properties which will vary according to its age, probably also according to its composition. If fed *ad libitum*, a third variable is introduced, in the difference between the appetite of individual guinea-pigs and consequently in the amount consumed.

In experiments destined to estimate the anti-scurvy value of any particular food stuff, *e.g.* milk in this instance, the foodstuff in question should constitute the only variable in the diet; if hay *ad libitum* is given with the basal diet, two and possibly three, variables are included in addition.

The inclusion of anti-scorbutic material in the basal diet makes it possible to protect guinea-pigs from scurvy with smaller rations of milk than could

otherwise be effected, and by such a procedure it might be possible to obtain a quantitative estimate of the comparative value of dried compared with raw milk, which could not be obtained by our method.

But the results of such experiments can only be regarded as trustworthy when the included material is standardised both as regards its own anti-scorbutic value and the quantity consumed by the guinea-pigs.

It is stated by Hess and Unger [1919, p. 295] that Chick and Hume and other workers at the Lister Institute have used dried milk as part of their basal dietary—"because it was found that this milk was devoid of anti-scorbutic power." We have never used dried milk as part of our basal diet in this Institute, nor do we regard it as devoid of anti-scorbutic power as the present paper shows clearly. The milk which has been used regularly in our basal diet has been autoclaved at 120° for one hour and given in constant quantity, 60 cc. daily to each guinea-pig. In milk so treated we have been unable to find any significant survival of anti-scorbutic value.

In the paper by Hart, Steenbock and Smith [1919] to which attention is also drawn, hay *ad libitum* was again included in the basal diet of the guinea-pigs and the same criticism applies. They too obtain protection from scurvy with rations of milk considerably smaller than those which we have found necessary: in the case of fresh milk a daily ration of 84 cc. gave complete protection from scurvy and partial protection was obtained with 30 cc. daily. The authors themselves [1919, p. 308] suggest the probability that "the hay still contains some of the anti-scorbutic vitamine, which in addition to that in the milk and grain is sufficent to prevent the development of the disease" on a ration of fresh milk smaller than would be needed to protect when no hay is given.

EXPERIMENTS WITH MONKEYS, BY E. MARGARET HUME.

Scurvy in monkeys has been described by Hart and Lessing [1913], Harden and Zilva [1918], and Chick, Hume and Skelton [1918, 3]. When these animals are fed upon a diet deficient in anti-scorbutic material but otherwise adequate, scurvy is developed in about ten weeks [Harden and Zilva 1918], with symptoms closely resembling those of human scurvy. Such are sponginess and bleeding of the gums with loosening of the teeth, and haemorrhages under the periosteum and in almost any part of the body. There is weakness and ultimately almost complete powerlessness of the hind limbs, a condition characteristic of infantile scurvy which has been called "pseudo-paralysis" [Barlow 1894], and which is apparently the result of haemorrhages into the hip and knee joints. The bones also become fragile and there is enlargement and disruption of the rib-junctions. When the disease is far advanced the whole body and limbs are tender.

Barlow ascribes the sub-periosteal haemorrhage in scurvy of infants to an increased vascularity of the periosteum from which blood is assumed to

escape and to collect between the periosteum and the bone. From post-mortem examinations of slight and incipient scurvy in monkeys it has seemed rather as if the bone became rarified at an early stage of the disease and that blood oozed outwards through the bone, presumably from haemorrhage in the bone marrow.

Alterations in the gums were observed to occur earlier and to be much more severe in monkeys which were changing their temporary for their permanent dentition; the onset of scurvy arrested the eruption of the teeth, the gums became swollen and blood blisters were frequently formed over the site of the budding tooth. Gum symptoms were usually the first sign of scurvy to be observed, but occasionally the weakening of the hind legs preceded them. This so-called "pseudo-paralysis" is very characteristic. At first only a slight decrease in activity is noticed, the animal does not jump as high or as carelessly as it was wont to do; this condition progresses until at last the hind legs become useless and the animal swings itself about in a sitting posture by the aid of its fore-limbs.

The effect of scorbutic symptoms upon the weight of the monkey is similar to that observed in case of guinea-pigs. About the time of the first appearance of scurvy symptoms, the animal ceases to gain in weight and soon afterwards a slow but gradually accelerated loss of weight begins (Fig. 3, curve A, p. 321). If a cure is then administered, this loss is soon checked and after a short period of maintenance weight is again put on at a remarkable rate (Fig. 3, curve A, p. 321). The original weight of the animal is regained and the normal rate of growth, if the animal is young and growing, is re-established.

The monkeys used in this set of experiments were not very numerous and were of various kinds and sizes. All were young, growing, healthy animals at about the age of the second dentition, probably, therefore, from one-and-a-half to three years old. They included the genera Macacus (two species), Cercopithecus (two species) and Cercocebus (one species). As the animals were so few and so various, special precautions had to be taken to avoid error due to idiosyncrasy. The method pursued was, in the first place, to determine the minimum dose of fresh and dried milk respectively, which would protect a monkey from scurvy when on a diet otherwise devoid of anti-scorbutic material. In the second place, when scurvy had developed upon a certain daily ration of dried milk, cures were attempted by substituting the same sized ration of raw milk. This procedure eliminated many sources of error, in that the comparison between the two foodstuffs was carried out upon the same individual animal.

The basal diet. The staple item was boiled polished rice, of which 100 g. (dry weight) was allowed daily to each animal; to this was added 30 g. daily of raw wheat germ to provide an abundant supply of the water-soluble B or anti-beriberi factor. A piece of wheaten biscuit, a few pea-nuts or dried peas or a little paddy rice were given as a relish. To the basal diet was added

the measured ration of milk, warmed very slightly and heavily sweetened; this served as the source of the anti-rachitic and fat-soluble A factor and was also the only source provided of the anti-scorbutic factor. When the ration was below 200 cc. a day it was made up to this amount by addition of milk autoclaved at 120° for one hour to deprive it of anti-scorbutic vitamine. This addition was made in order to increase the amount of anti-rachitic factor in the diet; the monkeys, however, would not always accept it.

The milk ration was usually divided into two parts and offered to the animals morning and evening, the monkeys being trained to drink out of a dish held in their cages. It was occasionally necessary to feed with a pipette, syringe or spoon if the animal grew tired of the milk and had to be coaxed to carry the experiment through.

The milk used for the experiments with raw milk was the same as that used in the work with guinea-pigs (p. 308). The dried milk was also of the same brand as in the foregoing experiments, but the work was confined to the English material of recent manufacture; in some trials it was one to two weeks old and in others two to three weeks old.

A summary of the results obtained is set out in Table II and full protocols of all the experiments are given later, p. 320. They include three trials with dried milk and six trials with raw milk. One of the latter, Exp. 1 *A*, was a curative experiment following a period on dried milk during which definite and severe scurvy was developed.

As regards the amount of raw milk which must be consumed daily to prevent scurvy in monkeys, rations of 50 cc. and 75 cc. proved too small in case of two monkeys, David (initial weight 2080 g.) and Diana (initial weight 2770 g.) respectively. 125 cc. was found adequate (see Exp. 6, Imp, weight 1940 g.), so also was 200 cc. (Exp. 8, Rags, weight 2230 g.), while 175 cc. cured Jane (Exp. 1 *A*, initial weight 2180 g.) of scurvy which she had acquired on a diet containing an equivalent amount of dried milk.

On the basis of these results we might place the minimum protective ration of raw cow's milk at about 100 cc. daily. The case of the monkey Chirgwin (Exp. 7) who developed slight symptoms of scurvy, but was otherwise in good health for six months upon a daily ration of 150 cc., is somewhat out of line with the above. This particular experiment was of less value than some of the others from the fact that the raw milk experiment followed a period of 43 days on dried milk, after which the nature of her ration had to be changed owing to her sudden, but persistent, refusal to take dried milk.

In the case of dried milk we have definite proof of the inadequacy of amounts equivalent to 175 cc. and 200 cc. milk daily in case of monkeys Jane (Exp. 1) and Toby (Exp. 2) respectively. Both these monkeys developed definite and severe scurvy in from three to four months; and in case of Toby, the ration was increased to 300 cc. without in any way relieving the scurvy symptoms which, on the contrary, became progressively more severe. The third monkey, Caesar (initial weight 2740 g.), received the

equivalent of 250 cc. daily. After about four months his condition suggested most distinctly the onset of scurvy, his ceaseless activity became less marked, his hind limbs became slightly weak and his weight stationary. As the time progressed, his condition improved, however, and it appeared as if his milk ration was increasing in anti-scorbutic value. The change for the better took place about the middle of June, about four weeks after incipient scurvy had been detected, and at a period of the year shortly after the time when cows change their winter stall feeding for the open pasture. Such an alteration in diet may be expected to influence the anti-scorbutic value of the milk given by the cow and also of the dried milk prepared therefrom, especially when, as was the case in these experiments, it is consumed very shortly after manufacture. A disturbing factor is thus introduced into the experimental work, when the series of experiments extends over one year as was the case in the present research. The milk was consequently derived from cows which during a portion of the time were stall-fed and during other portions were pasture fed. The milk, either dried or fresh, cannot therefore be regarded as a uniform article throughout the period. It is judged, however, that this fact did not materially alter the trend of the experiments, seeing that trials with raw and dried milk went forward simultaneously, and the latter was given so soon after manufacture. In the case of the monkeys Caesar and Chirgwin (see below, p. 323), however, the taking of the cows out to pasture about the middle of May was doubtless the means of raising a ration of dried milk of 250 cc. (Caesar) and a ration of fresh milk of 150 cc. (Chirgwin) from a non-protective to a protective amount.

The influence of differences in the cow's diet upon the anti-scorbutic value of the milk given makes it, however, impossible to obtain an absolute standard for the anti-scorbutic value of cow's milk and it is only possible to say that *the daily ration of raw milk which protects from scurvy a monkey of* 2—3 *kg. weight is from* 125 *to* 175 *cc., while the corresponding ration of dried milk, of fresh manufacture, is from* 250 *to* 300 *cc.*

It is interesting to note that the amount of raw milk needed to protect a guinea-pig from scurvy, 100 to 150 cc., is almost as great as that needed to protect a monkey. The corresponding amount of dried milk, freshly manufactured, necessary for a guinea-pig, would therefore be the equivalent of well over 200 cc., an amount far too large to be consumed by an animal of 300 to 400 g.s weight. It is not, therefore, surprising that all efforts to protect guinea-pigs from scurvy with dried cow's milk, even when given in a concentration greater than the normal, should have proved unsuccessful.

Experiment with heated milk.

Only one experiment was made in which the anti-scorbutic value of raw cow's milk, scalded, was tested.

The experiment was designed to show whether milk, heated to a degree

which is commonly believed to destroy the bacillus of tuberculosis, suffers serious loss in anti-scorbutic vitamine.

The milk was heated quickly in an enamel pan over a Bunsen burner; it was removed at the moment when it frothed up, at the boil, and was allowed to cool in the air. The total period of heating was found to be about four minutes, during which time the milk was between 70° and 100° for about one-and-a-half minutes. After the pan was lifted from the flame, four minutes elapsed before the temperature of the milk fell again to 70°, and a further 17 minutes before it reached 40°. The whole operation consisted therefore in exposure to a temperature between 70° and 100° for about five-and-a-half minutes.

A ration of 200 cc. of such milk was given to a monkey (Toby) which had developed scurvy on a daily ration of 200 cc. of dried milk. The cure was rapid and complete.

The loss in anti-scorbutic vitamine is therefore not as great as is the loss suffered by milk in drying, but since the minimum amount of raw milk needed to protect a monkey is less than 200 cc., being about 150 cc., the milk might, in the scalding, have suffered loss to the extent of 25 per cent. of the anti-scorbutic vitamine it originally contained, without its being possible to detect such loss by the one experiment made. A loss of more than 25 per cent. would however have reduced the anti-scorbutic value of the ration to less than that of 150 cc. raw milk, but the rapidity of the cure suggested that the animal was receiving a considerable excess over and above the minimal ration needed to protect. Such scalding may therefore be looked upon as a means of sterilisation causing comparatively little damage to the anti-scorbutic vitamine.

Growth promoting powers of raw, dried and heated milk.

None of the experiments described was specially designed to test the growth promoting powers of the milks used, but as in most cases the milk ration was, at the same time, the chief, if not the sole source of the fat-soluble A (anti-rachitic) growth promoting factor, it is possible to draw certain deductions. These are necessarily somewhat rough owing to the diversity in kinds and ages of the monkeys used.

It was found that a ration of 200 cc. daily of milk of any description, raw, dried, scalded or autoclaved, gave good growth but where smaller quantities were used growth was retarded.

In the post-mortem examination of the monkey, David (Exp. 4), which received 50 cc. or less of raw milk, distinct symptoms of rickets were observed. The rib junctions were enlarged and there were also secondary enlargements on the bony shaft of the rib, enclosing cartilaginous islands (see Fig. 2), such as are so frequently seen on the ribs of monkeys kept long in confinement on unsuitable diet in zoological gardens. This monkey although

Table II. *Anti-scorbutic value of raw, dried and heated cow's milk. Experiments with monkeys.*

	No. of exp.	Period of year	Name of animal	*Average daily ration, cc.	Time of exp. days	Time after which onset of scurvy noted, days	Initial weight of animal g.	Change in weight during course of experiment, %	Remarks
(*a*) Dried Milk of fresh manufacture 2–3 weeks old	1	July 1918 to December 1918	Jane (*Macacus cynomolgus*)	175	142	105	2090	+ 4	Cured with equivalent amount of raw milk before any great loss in weight had occurred (Exp. 1 *A*)
	2	Dec. 1918 to March 1919	Toby (*Cercocebus fuliginosus*)	1st period 200 2nd period 300	97 7	87 continuous	2470	+10·5	Cure attempted with 300 cc. dried milk, symptoms worse after 7 days (see also Exp. 2 *A*)
	3	Feb. 1919 to July 1919	Caesar (*Cercopithecus callitrichus*)	250	161	117 but disappeared later	2740	+15·5	Scurvy symptoms observed on 117th day, remitted later about time when cows go to pasture
(*b*) Raw Milk	1 *A*	Dec. 1918 (1) to Feb. 1919, (2) to July 1919	Jane (*Macacus cynomolgus*)	1st period 175 †2nd period 125	54 162	— —	2180 2450	+12 +14	Successful cure of scurvy developed on similar ration of dried milk Excellent health and growth
	4	July 1918 to Dec. 1918	David (*Macacus rhesus*)	50 (*maximum value*)	150	66	2080	−40	Scurvy developed on full ration of 50 cc. daily, later part of ration refused; died from dysentery, signs of mild scurvy and rickets at post-mortem examination
	5	July 1918 to Dec. 1918	Diana (*Macacus rhesus*)	75	143	119	2770	+ 7	Gained in weight until onset of scurvy, refused part of ration with increase in severity of symptoms; successful cure with 10 g. daily germinated peas
	6	Dec. 1918 to July 1919	Imp (*Cercopithecus sabaeus*)	†125	182	—	1940	+20	Excellent health and fair growth
	7	Jan. 1919 to July 1919	Chirgwin (*Cercopithecus sabaeus*)	150	184	115 (diminished later)	2770	+10·5	Previous to experiment received ration of dried milk equivalent to 200 cc., imperfectly taken, for 43 days. Scurvy symptoms on 115th day but much mitigated when cows go to pasture
	8	Dec. 1918 to July 1919	Rags (*Cercocebus fuliginosus*)	200	225	—	2230	+50	Excellent health and growth
(*c*) "Scalded" Milk	2 *A*	March 1919 to May 1919	Toby (*Cercocebus fuliginosus*)	200	49	—	2730	+11	Successful cure of scurvy developed on dried milk, marked improvement after 7 days (see Exp. 2 above)

* In case of dried milk expressed as the equivalent volume of fresh milk.

† 75 cc. autoclaved milk added from the 60th day in Exp. 1 *A* 2nd period, and from 105th day in Exp. 6.

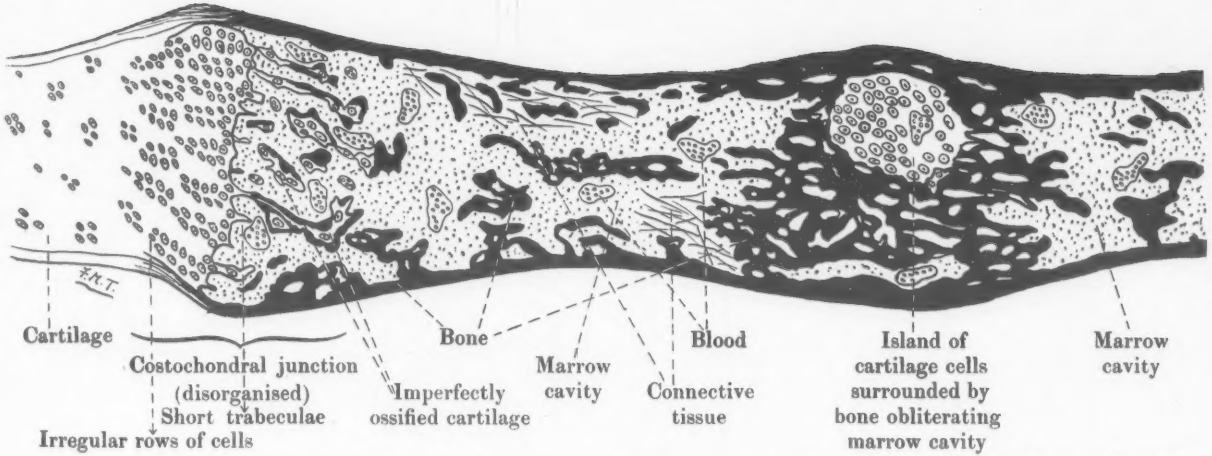

Fig. 2. Diagrammatic representation of a section of a rib junction and secondary nodule believed to be of rachitic origin (Monkey 4 David). Distance of nodule from junction 1·1 cm.

a young and growing one (initial weight 2080 g.) showed a daily increment of 1·5 g. only, up to the time when he developed scurvy. The histology was kindly examined by Miss F. M. Tozer, to whom I am indebted for the drawing reproduced in Fig. 2.

If a comparison is made between the weight curves (Fig. 3) of the monkeys Toby (curve A, 200 cc. dried milk) and Rags (curve B, 200 cc. raw milk), for the first 70 days of the experiment, *i.e.* up to the time when Toby developed scurvy, it is seen that Toby on dried milk shows a daily increment of 6·4 g. while Rags on raw milk shows one of 6·1 g. The rate of growth is therefore almost the same, being slightly in favour of the monkey on dried milk. These two animals are particularly well chosen for comparison as being more nearly alike than any of the other experimental monkeys. Both were male *Cercocebus fuliginosus*, purchased together, presumably of the same past history, and their initial weights, 2470 g. and 2230 g. were also nearly the same.

No two of the other animals are strictly comparable either in themselves or in the ration they received.

Jane (initial weight 2090 g.) over the various periods of her experiment gives the following daily increments:

Daily ration of milk	Days of exp.	Rate of growth, g. per day
Dried milk, 175 cc.	63	4·1
Raw milk, 175 cc. (period of cure) ...	24	2·5
,, 125 cc.	60	2·0
,, 125 cc.; autoclaved milk, 75 cc.	102	2·2

The only marked superiority here, seems to be in the case of the large ration of dried milk; the large ration of raw milk was not continued sufficiently long to give a conclusive result. In considering these figures it must, however, be remembered that monkeys' growth is not necessarily uniform and conclusions drawn from comparative growth, over consecutive periods, on the same animal may not give reliable results. As far as the experiments go, however, the results obtained with monkeys confirm those obtained with guinea-pigs and show that dried milk is not appreciably inferior to raw milk as regards the growth promoting properties, and the content of the fat-soluble A factor. Experiments with rats [see Coutts 1918; Lane Claypon 1916] are in harmony with this view.

PROTOCOLS.

David. (No. 4, *Macacus rhesus*. Male. Initial weight 2080 g.) Raw milk ration 50 cc. Scurvy developed.

For the first 42 days he refused to drink the milk, it was therefore poured over the cooked rice meal; the amount consumed was consequently less than 50 cc. per diem.

For the next 41 days the milk was taken regularly from the syringe. About the 66th day haemorrhagic spots were noticed on the gums and incipient scurvy was diagnosed. On the 85th day the animal met with an accident, biting its tongue so severely that it refused its milk and never again consumed the full 50 cc. The scurvy symptoms progressed, stiffness of the hind legs

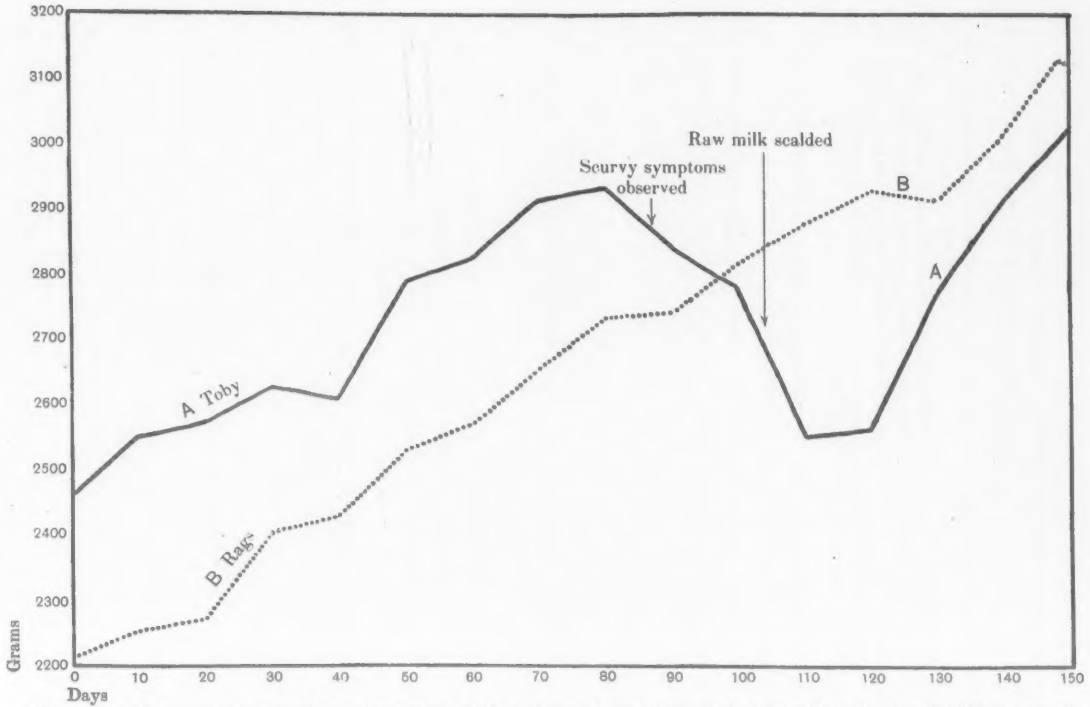

Fig. 3. Weight curves of two monkeys, showing comparison between fresh milk scalded, dried milk, and raw milk. Toby (curve A) received 200 cc. dried milk daily for 104 days, scurvy developed on 87th day. Fresh milk scalded (200 cc.) daily administered from 104th day with complete cure.

Rags (curve B) received 200 cc. raw milk daily throughout and remained in health.

was observed, the animal lost weight and finally severe dysentery set in; he was chloroformed on the 148th day.

The post-mortem examination showed incipient scurvy, haemorrhage into the synovial cavities of knees, elbows and hips, and petechial haemorrhage in the dura mater; there was sponginess at the ends of the long bones and incipient sub-periosteal haemorrhage in the same regions. There was enlargement of the costochondral junctions, probably of rachitic origin, and there were also secondary enlargements along some of the rib bones, nearer to the spine (see Fig. 2, p. 319). The bones of the ribs and skull were much rarified and could be cut through easily with scissors.

The large intestine showed the inner surface mucoid and slightly haemorrhaged, probably from dysentery, but no organism was recovered which corresponded to a typical dysentery.

Scurvy symptoms first appeared when the full ration of 50 cc. of raw milk was being consumed. A moribund state was induced by dysentery supervening, presumably on a condition where the immunity was lowered by incipient rickets and scurvy.

Diana. (No. 5, *Macacus rhesus*. Female. Initial weight 2770 g.) Raw milk, average consumption 75 cc. Scurvy developed.

This monkey consumed on an average 75 cc. raw milk daily from the syringe and on this diet she gained weight slowly till scurvy symptoms supervened. Slight sponginess and haemorrhage of the gums were observed on the 119th day. From this time onward the milk was consumed less well and the scurvy symptoms progressed more rapidly; the gums became markedly spongy and bleeding; the hind legs became uncertain and a haemorrhage about the eye produced the discoloration characteristic of a black eye.

On the 143rd day, anti-scorbutic material in the form of 10 g. daily of raw germinated peas (soaked 24 hours, germinated 48 hours) was administered together with a liberal ration of autoclaved milk. A gain in weight soon became apparent and the scorbutic symptoms disappeared.

An average consumption of 75 cc. raw milk a day was therefore insufficient to protect this animal from scurvy.

Jane. (No. 1, 1*A*, *Macacus cynomolgus* (*fasciatus*). Common macaque. Female. Initial weight 2090 g.) Dried milk, average daily ration equivalent to 175 cc. Scurvy developed and was cured later by an equal ration of raw milk.

This monkey began to show haemorrhage of the gums about the 105th day and leg weakness appeared soon after. About this time the change from primary to secondary dentition took place. On the 96th day one of the upper median incisors was lost and at first the new budding tooth could be seen in the gap; soon however the gum became swollen and purplish and a haematoma developed over the new tooth.

The monkey continued to take her milk well and did not lose weight seriously. The symptoms progressed in the gums which became much swollen and of a dusky purple colour; the haematoma continued unchanged and the new tooth remained uncut. The legs became very weak and almost useless.

On the 142nd day the ration was changed to one of fresh milk and in order to make the comparison perfectly fair, the quantities given were exactly the same in inverse order as had been the quantities of dried milk consumed. Improvement was rapid. In ten days the animal was much more active and weight was put on speedily; in 20 days the haematoma was almost gone and the cutting of the tooth, delayed for over nine weeks by the attack of scurvy, proceeded normally.

After the cure had proceeded for 54 days and was deemed to be complete, the ration of fresh milk was cut down to 125 cc. daily. Good health and fair growth were maintained but on the 68th day of this treatment, it was decided to raise the ration to 200 cc. by adding 75 cc. of autoclaved milk. In this way it was intended to increase the ration of the fat-soluble A growth factor, while keeping the supply of the anti-scorbutic material the same. This change seemed to lend a slight impetus to growth but it seems probable that the monkey was in any case a slow growing one.

After 162 days the animal was still in good health and it was concluded that in this case 125 cc. daily of raw milk provided sufficient of the anti-scorbutic factor, while 175 cc. of dried milk had failed to do so.

Imp. (No. 6, *Cercopithecus sabaeus*. Male. Initial weight 1940 g.) Raw milk ration 125 cc. Protection from scurvy.

This monkey received 125 cc. raw milk as its sole source of the anti-scorbutic factor for 182 days and showed no sign of scurvy throughout.

On the 60th day 75 cc. autoclaved milk was added as the animal, although in excellent condition, was occasionally subject to convulsions. It was thought that these might be of rachitic origin and it was decided to give an additional supply of fat-soluble A in this manner. The convulsions ceased but the rate of growth was never rapid, 125 cc. raw milk daily however seemed to supply enough of the anti-scorbutic factor as no symptom of scurvy was ever observed.

Chirgwin. (No. 7, *Cercopithecus sabaeus*. Female. Initial weight 2770 g.) Dried milk ration 200 cc. for short period, followed by raw milk ration 150 cc. Result inconclusive.

This monkey started under somewhat unsatisfactory conditions and never cooperated well at any time. It was originally intended that she should receive a ration of 200 cc. of dried milk which she took more or less regularly for 40 days; from that period she refused to touch it and the experiment had to be altered to one with a daily ration of 150 cc. raw milk. This amount was regularly consumed, for any lapses were made up by proportional increase on succeeding days.

Growth was very slow but the animal refused to drink an additional 50 cc. of autoclaved milk which was offered in order to raise the total volume to 200 cc. and to afford an increased supply of fat-soluble growth factor. On the 104th day of the raw milk experiment a slight redness of the gums was noticed, which increased. On the 120th day a large fluctuating swelling on the crown of the head, which had been noticed for several weeks, had become so large that it was decided to operate. No pus was found, only liquid blood which was drained away and the incision sewn up. Such a haematoma was probably not formed traumatically, on account of its slow growth, and it is concluded that the haemorrhage was due to scurvy, a conclusion confirmed by the slightly scorbutic condition of the gums. Contrary to expectation the wound did not fill up again with blood, but healed satisfactorily and at the same time the gums also regained their normal healthy appearance.

The opening of the haematoma took place on May 17, 1919, and on enquiry it was ascertained that May 13th was the date upon which the herd, whence the fresh milk was derived, had been turned out to pasture. The conclusion drawn, therefore, is that the alteration in diet of the cows had raised the anti-scorbutic value of the milk from a ration below, to a ration just above, the protective minimum. The preliminary feeding on dried milk had ended 15 weeks before any scorbutic symptoms began to show, this may have had an influence upon the subsequent history, but it seems unlikely.

The ration of 150 cc. raw milk was continued till the 188th day when the experiment was terminated; the animal seemed in excellent health, there were a few minute bleeding points on the gums, however, which may have been of scorbutic origin.

The conclusion to be drawn from this experiment seems to be that 150 cc. raw milk was in this case just on the borderline of protection, the discrepancy between this result and that of the two preceding experiments, where Jane and Imp were protected with 125 cc. raw milk daily, is doubtless due to individual idiosyncrasy and also possibly to differences in the size of the animals, for the animal at present under consideration was considerably larger than either of the other two.

Caesar. (No. 3, *Cercopithecus callitrichus*. Male. Initial weight 2740 g.) Dried milk equivalent to 250 cc. Result inconclusive.

This animal was fed a ration of dried milk equivalent to 250 cc. daily: growth was fairly good until the 88th day, when it ceased. About the 113th day, the animal's activity became

diminished and very slight redness of the gums was apparent. These symptoms continued and the animal became progressively less active with slightly falling weight until the 130th day, when the weight began to rise again, activity was slowly restored and the slight gum symptoms disappeared.

Such a mild attack of scurvy and spontaneous cure without any intentional alteration in the diet or the amount of it taken, is probably to be explained in the same way as with Chirgwin, the preceding monkey. In that case it was attributed to alteration in the properties of the raw milk ration, consequent upon the taking of the cows out to pasture with corresponding alteration in the antiscorbutic properties of their diet and of the milk given by them. In Caesar's case the date of his sickening and recovery was early in June 1919, but as the dried milk used was two to three weeks old, it would probably have been milked in the beginning or middle of May, *i.e.* just about the time that the cows supplying our fresh milk were proved to have changed their habits and diet for the summer. The manufacturers of the dried milk were applied to for information concerning the herd from whose milk the dried milk was prepared, but they were unable to make any definite statement with regard to dates, since the dried milk was prepared from material furnished by a great number of farms. It is, however, a fair assumption that the majority of cows would go to pasture early in May.

It may be concluded from the results of this experiment, therefore, that 250 cc. dried milk, freshly prepared, is about the minimal protective ration for a monkey weighing from two to three kgs.

Rags. (No. 8, *Cercocebus fuliginosus*. Male. Sooty Mangabey. Initial weight 2230 g.) Fresh milk, daily ration 200 cc. Excellent health and growth.

This animal and the next, Toby, form an excellent contrast as they were similar in every particular, save only for the difference in the daily ration of milk fed to them. Their weight curves are set out for comparison in Fig. 3, p. 321.

Rags received 200 cc. raw milk daily for 225 days during the whole of which time he remained in perfect health and added 50 % to his weight. No trace of any symptom of scurvy was ever observed. At the close of the experiment he weighed 3000 g. but notwithstanding his size, 200 cc. daily was a ration ample to protect from scurvy and to promote good growth.

Toby. (No. 2, *Cercocebus fuliginosus*. Male. Sooty Mangabey. Initial weight 2470 g.) Dried milk, daily ration equivalent to 200 cc. Severe scurvy developed, and was cured upon an equivalent ration of fresh milk, scalded.

This animal was fed 200 cc. daily, and throve and grew well for about 70 days (Fig. 3, Curve A). After the 70th day growth slackened and it was noticed that there was a slight diminution in the animal's agility. By the 77th day, the symptoms were definite, the gums were swollen and purple and the hind limbs showed distinct weakness. The symptoms still progressed and the monkey lost flesh rapidly. On the 97th day he could not walk at all but was only able to swing himself in a sitting posture and his gums bled freely. On this day it was decided to attempt a cure by increasing the daily ration to 300 cc. of dried milk. The amount was taken readily and continued for a week, but the weight still fell and the general condition became worse.

On the 104th day the ration was changed to 200 cc. of fresh milk which had been "scalded" (see p. 317). The day after this treatment was commenced a slight improvement was detected, in four days the animal could scramble out of his cage and in a week was markedly better. The fall in weight was, however, not arrested for six days and a definite rise in weight did not set in for over a fortnight, when the monkey began to gain in weight with astonishing rapidity. In 16 days after the cure began, he could run about; in 62 days after, he was deemed to be completely recovered and the experiment was ended. Dried milk equivalent to 200 cc. therefore was insufficient, and 200 cc. raw milk, scalded, was amply sufficient to protect this animal from scurvy.

Application of the foregoing results to the feeding of infants.

At the present time dried milk is being used for the feeding of infants to an ever increasing extent and it cannot be denied that it offers many advantages over ordinary raw milk both in general convenience and in absence of risk from tuberculosis and other infections. The question arises as to whether its nutritive properties are equal to those of raw fresh milk, or boiled fresh milk, and, seeing that its anti-scorbutic value has definitely been proved to be lower, whether this defect is of serious importance, when it is employed for the nourishment of infants.

Many infant specialists having large experience of feeding with dried milk declare that they have never encountered a case of scurvy among the children fed upon it [see Lane Claypon 1916]. It is not, however, stated always for how many months an exclusive diet of dried milk was continued nor whether all extra anti-scorbutics were excluded. It must also be remembered that slight scorbutic defects may exist without giving rise to the classic appearance of severe infantile scurvy and may even escape detection altogether. Such conditions have been observed by Hess and Fish [1914] and by Miller [1917]. But in absence even of such mild symptoms as were described by these workers, there might yet be conditions which were unsatisfactory as regards the health of the infant, due to an insufficient supply of the anti-scorbutic material in the diet. The experimental work upon scurvy has shown that there may be defects in the structure of bony tissues, only discoverable by microscopical examination, when there has been only a slight deficiency of anti-scorbutic material in the diet, and when no macroscopic signs of scurvy have been detected during life or post-mortem [see Delf and Tozer 1918; Zilva and Wells 1919]. For example, it is during the first months of an infant's life that processes are at work upon the construction of the permanent teeth, and any defect in the structure of these may not be apparent until many years later. It is probable, therefore, that an entirely trustworthy estimate of the anti-scorbutic value of dried milk as the sole food during infancy cannot be formed until this point has been investigated.

The results of the experimental work leave no doubt that dried cow's milk is inferior to raw milk in anti-scorbutic properties and that raw cow's milk itself is a food of comparatively poor value in this respect. It is reasonable to assume that in a diet of raw cow's milk there is not any great excess of anti-scurvy vitamine above the infant's requirements and that when dried milk is substituted, there is risk that the supply of this vitamine may fall below what is necessary for complete safety. Under these circumstances the only wise course is to provide a supplementary anti-scorbutic.

With regard to the growth promoting properties of milk, and in particular its content of the fat-soluble accessory factor, no evidence was obtained from the present work, either from the experiments with guinea-pigs or from those with monkeys, that any loss is suffered during the process of drying. The

results of a separate series of experiments upon this point by one of us (R.E.B.) are shortly to be published. The results of this work also go to show that the growth promoting value of fresh milk and dried milk are roughly about equal. If, as seems probable, from the work of E. Mellanby [1918, 1919], the fat-soluble growth factor and the anti-rachitic factor prove to be the same, then whole dried milk can be relied upon to possess the same value as fresh milk for maintenance of growth and for prevention of rickets. As regards the water-soluble B accessory growth factor, its presence in abundance in many dry foodstuffs indicates that dried milk may be assumed to possess it in about the same amount as fresh milk. As a matter of fact none of the foregoing experiments was designed to give information upon this point, as the water-soluble or anti-neuritic factor was provided also in the grain ration of the experimental animals. The question is of importance, however, when, as is the case with infants, the diet is composed exclusively of milk.

There is urgent need for experimental work in which comparison is made between human milk and cow's milk in respect of the content of the three accessory factors, (1) anti-scorbutic, (2) fat-soluble (or anti-rachitic), (3) water-soluble or anti-neuritic factor. There is at the present time no direct information upon this point and such results would be of great value in helping to settle many of the problems of infant feeding. The obstacle to such work is the great difficulty in obtaining a regular supply of human milk for the long period of time required for trustworthy experiments of this type.

On theoretical grounds we should expect the milk of pasture-fed cows to be richer in the first two of the above vitamines, seeing that a diet of green leaves is a rich source of both. Moreover cow's milk is constituted to be the sole nourishment of the calf, an animal of much quicker growth than the human infant, and therefore presumably needing a more abundant provision of all diet constituents, including the vitamines. On such a basis of argument summer milk from cows may be supposed to contain an amount of accessory factors in excess of the requirements of the human baby. On the other hand, it must be remembered that human milk has important advantages in that it is always taken in the raw condition and undiluted, and that the above arguments in favour of cow's milk refer only to the milk given on the natural diet of grass. In the winter stall-fed cows commonly receive an artificial diet of hay, roots and oil-cake prepared from seeds, which is a diet deficient in the fat-soluble growth factor and, especially if the roots consist of mangolds and not of swedes or turnips [see Chick and Rhodes 1918], deficient also in the anti-scorbutic factor. The milk given will show corresponding deficiencies.

Seeing therefore that cow's milk is a foodstuff of inconstant value as regards its anti-scorbutic properties and that the latter are much reduced in the process of drying, there is every reason to advocate the use of an extra anti-scorbutic in the dietary of infants nourished on dried milk. The results of experiments specially carried out to determine the most suitable substances

for this purpose have been published by Chick and Rhodes [1918], working in this Institute, and among the foodstuffs investigated, raw orange juice and raw swede juice were specially recommended both for potency and for general acceptability. Hess and Unger [1918] have recommended the use of the juice of raw or tinned tomatoes, and their results, as regards tinned tomatoes, have since been confirmed by Chick and Rhodes [unpublished experiments].

Summary and Conclusions.

1. A series of nutritional experiments with guinea-pigs is described in which the diet consisted of oats and bran, and a ration of cow's milk constituted the only anti-scorbutic material. It was found possible to prevent scurvy and to maintain good health in animals of 300 to 400 g. weight if 100 to 150 cc. of raw cow's milk were consumed daily. When dried milk was substituted for the fresh milk scurvy was not prevented even when amounts equivalent to these were taken. This was true whether the dried milk was of recent manufacture (one to two weeks old) or of much greater age (six to twelve months).

2. A similar result was obtained in a series of experiments with monkeys. In this case only dried milk of recent manufacture was used. Rations of fresh milk of 50 to 75 cc. daily were not sufficient to prevent scurvy in young animals of 2 to 3 kg. weight; a ration of 100 to 150 cc. daily was however adequate. In case of dried milk the equivalent of about 250 to 300 cc. (at least) was necessary. In one instance severe scurvy, developing on an average daily ration equivalent to 175 cc., was cured rapidly and completely by substituting an equal ration of raw milk. In another case, where scurvy developed on an average daily ration of dried milk equivalent to 200 cc., increase of the ration to the equivalent of 300 cc. caused no improvement, but a cure was successfully effected with a daily ration of 200 cc. "scalded" milk, *i.e.* raw milk quickly brought to the boil and immediately set to cool (exposure to a temperature between 70° and 100° for about five-and-a-half minutes).

3. No indication was obtained from either series of experiments that the growth promoting properties of fresh milk suffer any serious damage during the process of drying.

4. Evidence is brought forward indicating that summer cow's milk has a higher anti-scorbutic value than that given in the winter. This is to be explained by the difference in diet, from fresh herbage in the summer, to hay, oil-cake, cereals and roots in the winter. The use of turnips or swedes, rather than mangolds, should result in a milk of higher anti-scorbutic value.

5. The bearing of these results upon infant feeding is discussed and arguments are given in favour of employing an extra anti-scorbutic in the diet of infants nourished upon dried cow's milk. For this purpose the raw juices of oranges, swedes, and tomatoes are specially to be recommended and in case

of the last named, good results have been obtained even with the tinned material.

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